Lymphatics and lymphatic-like structures in melanoma

A pathobiological study

Ruud Clarijs

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Contents

1 Introduction

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Cancer is a common cause of death in Western society. A malignant tumor can arise in any organ and as long as it is confined to its organ of origin, patients can often be cured through surgical removal of the tumor. However, many cancers spread to other sites in the body and metastasis is the leading cause of death in cancer patients. Tumor cell extension can occur by different mechanisms such as direct invasion of surrounding tissues (per continuitatem), spread through body cavities, spread through lymphatic vessels (lymphatic metastasis) and via blood vessels (hematogenous metastasis). Despite its clinical relevance, the mechanisms leading to metastasis via the blood stream are only partly understood, and even much less is known about metastasis via lymphatic vessels. In addition, it appears that metastasis is not strictly dependent on dissemination via blood and lymphatic channels, but that also certain extracellular matrix deposition patterns may be involved. Therefore, we focused in this thesis on the role of the microvasculature and extracellular matrix patterns in progression and metastasis of human cancer with emphasis on cutaneous melanoma and, especially, uveal melanoma.

Involvement of blood and lymphatic vessels in tumor growth and metastasis

Solid tumors need blood supply for nutrition and growth.[1] If a neovascular bed develops in and around the tumor, this process of angiogenesis is modulated by growth factors secreted by tumor cells or by an inflammatory infiltrate.[2,3] Next to facilitating tumor growth, the vascular bed is essential for metastasis. Indeed, in several types of tumors, a high mean vascular density was related to poor prognosis associated with a high rate of metastasis.[4] This relationship may be explained by a simple, but hypothetical principle: the more vessels, the greater the probability that tumor cells invade the vascular bed and escape from the site of origin of the tumor. However, besides the number of vessels present in the tumor, other properties will determine metastatic spread as well. For example, proteolytic and migratory activity, the expression of adhesion molecules and deposition of extracellular matrix by stromal and/or tumor cells contribute importantly to tumor growth and rate of metastasis.[5]

Many factors influence metastasis and could explain the aberrant findings in tumor types that do not obey the above-mentioned principle, like cutaneous melanoma and head-and-neck squamous cell carcinoma. In these types of malignant tumors, the microvascular density appears not to correlate with metastatic spread.[6-8] Until now, the reason for this lack of correlation is unclear but it may be speculated that, next to several properties of tumor cells and tumor-associated stromal cells, the presence of a lymphatic vascular bed is an important factor. It is obvious that the preference of a tumor to spread initially via the lymphatic vascular bed may be dependent on a high intrinsic lymphatic density in the tissue where the tumor arose.

Lymphatics can be invaded by tumor cells when the tumor reaches pre-existent vessels by outgrowth driven by angiogenesis or, alternatively, this might be accomplished by formation of new lymphatic vessels (lymphangiogenesis). In the first case, strong angiogenesis, reflected by high vascular density in the primary tumor, may increase lymphatic spread indirectly.[9] By the lymphatic route, metastases are then formed in locoregional lymph nodes. Outgrowth of tumor metastases on its turn could invade both neighboring lymphatic and blood vessels in lymph nodes. As a result of lymph

node metastasis, the thoracic lymphatic duct would collect lymphatic fluid containing tumor cells and drain it into the blood stream in the superior vena cava. Tumor cells could easily be disseminated by this route to the blood stream. Moreover, shunts that may exist between lymphatic and blood vessels in lymph nodes could further facilitate hematogenous spread. By this hypothetical mechanism, the lymphatic vasculature could contribute to the formation of distant metastasis and explain the lack of correlation between high blood vessel density and prognosis found in cutaneous melanoma. In the second case, there is the possibility that occurrence of tumor-induced lymphangiogenesis contributes importantly to local metastasis. Indeed, by increasing peri- and intratumoral lymphatic density, tumor cells may become in close relationship with the lymphatic vasculature.

Table I. Lymphatic markers and their expression pattern

Markers	Expression pattern
CD31	expression on blood vessels
Flt-4	re-expression on blood vessels in pathological conditions
podoplanin	expression in vascular tumors, kidney podocytes, osteoblasts and lung alveolar type I cells
LYVE-1	expression in normal kidney, pancreas, adrenal and thyroid epithelia and in hepatic blood sinusoidal endothelial cells
Prox-1	expression in the lens, heart, liver and nervous system
β-chemokine receptor D6	expression on a subset of the lymphatic vasculature
desmoplakin	expression in epithelia

Evaluation of lymphatic vasculature

Although in several studies blood and lymphatic vessels were discriminated by exploiting histo-morphological differences,[10-12] lack of reliable lymphatic endothelial markers (Table I) has thus far hampered a consistent evaluation of the lymphatic vasculature in both normal and tumor tissues.[13] Therefore, details of the anatomical relation between lymphatics and tumor cells on the one hand and the occurrence of lymphangiogenesis on the other, are essentially unknown. Recently, our group has developed a double staining protocol which differentially stains blood and lymphatic vasculature using the blood vessel endothelial marker PAL-E and the pan-endothelial marker CD31 in frozen sections of normal skin and cutaneous melanoma.[9] Another possible candidate marker for lymphatic immuno-staining is Flt-4 (also known as vascular endothelium growth factor receptor-3 (VEGFR-3)).[14] In adult life, Flt-4 is restricted exclusively to lymphatic endothelium.[14-17] However, recent studies demonstrated that this receptor is upregulated on blood vessels in carcinoma,[18,19] making this marker less useful for detection of lymphatics in tumors. Lymphatics may also be detected by an antibody directed against podoplanin.[20,21] Podoplanin is a membrane glycoprotein that was identified on podocytes in rat kidneys [22] and was expressed in endothelial cells of normal lymphatics and benign lymphatic tumors.[20] LYVE-1 (lymphatic vessel endothelial hyaluronan receptor), the ß-chemokine receptor D6 and Prox-I have been recently reported to be lymph-specific.[23-26] However, recent studies

showed that these markers may have their drawbacks as well (Table I).[25,27] Less well known antibodies that may be suitable for lymphatic detection are LyMAb [28] and a monoclonal antibody direct against desmoplakin-containing endothelial junctions.[29-31] Finally, it has been reported that lymphatics can also be visualized by 5'-nucleotidase enzyme histochemistry.[32-34]

Tumor-induced lymphangiogenesis

Initially, Folkman raised the question whether intratumoral lymphatics are present, newly formed, and functional and whether they are part of the tumor vascular system.[35] He asserted that tumors do not possess lymphatics which has lead to some discussion.[35-37] However, lymphatics present both intra- and peritumorally have been reported.[9] Although it could not be discriminated whether intratumoral lymphatics were pre-existent or newly formed, the occurrence of (tumor-induced) lymphangiogenesis in adults was initially suggested in the context of wound healing and head and neck cancer.[17,38] Further evidence of tumor-induced lymphangiogenesis in humans is provided by recent experimental animal studies.[39-44] As a result of these pioneering publications, the role of lymphatics and the possible occurrence of lymphangiogenesis in cancer has become a hot topic.[45-49]

It is not unlikely that the growth factors involved in vasculogenesis and lymphangiogenesis during embryogenesis (for review: see ref. 50) are also involved during tumor growth in adults, similar to what has been described for hemangiogenic factors. Members of the family of VEGFs are prime regulators of angiogenesis, via their interaction with the VEGF receptors Flt-1 (VEGFR-1) and KDR (VEGFR-2) (for extensive review: see ref. 51). In vessel walls in adults, Flt-1 and KDR are exclusively expressed on blood vascular endothelial cells whereas the third member of the VEGF-receptor family, Flt-4, is restricted to lymphatic endothelium.[14] The role of Flt-4 in normal lymphatic vascular function is established by studies demonstrating that hereditary lymphoedema is caused by missense mutations in the Flt-4 encoding sequence.[52,53] Furthermore, Flt-4 has been implicated in both angiogenesis and lymphangiogenesis during embryogenesis.[14] Overexpression of the ligand of Flt-4, VEGF-C [54,55] in the skin of transgenic mice resulted in lymphatic endothelial proliferation and vessel enlargement.[56] Other studies [39,57,58] pointed at a role of VEGF-C and the other ligand of Flt-4, VEGF-D [59] as a specific lymphatic growth factor. Indeed, overexpression of VEGF-C and VEGF-D have been shown to induce lymphangiogenesis in a number of different murine and avian chorioallantoic membrane xenograft models,[39-44] thereby pointing at an existence of tumor-induced lymphangiogenesis in human patients. This possibility is supported by the presence of both VEGF-C and Flt-4 expression [19,60-64] and a relationship between VEGF-C expression and lymph node metastasis in different human tumor types.[33,63,65-68] VEGF-A does not bind to Flt-4 and induces angiogenesis exclusively,[56] indicating that the receptor mediating putative lymphangiogenesis must be Flt-4. This hypothesis is indeed confirmed by recent studies: first, it appeared that a soluble form of Flt-4 is a potent inhibitor of VEGF-C/-D signaling. Soluble Flt-4 expressed in the skin of transgenic mice, inhibits fetal lymphangiogenesis and induces a regression of already formed lymphatic vessels.[69] Second, signaling via Flt-4 alone was sufficient for lymphangiogenic signals, since a mutated VEGF-C, which only

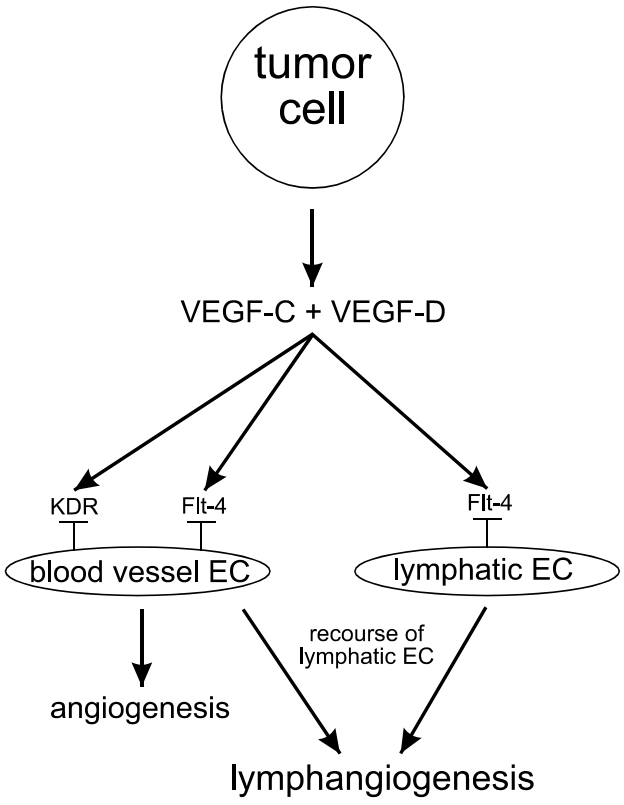

Figure 1. Schematic drawing of a hypothetical mechanism by which malignant tumors can induce lymphangiogenesis. Briefly, tumor cells express and release VEGF-C and -D. Both grow factors activate Flt-4 expressed on endothelial cells (EC) of both blood and lymphatic vessels. Both types of vessels could serve as possible sources of lymphatic endothelial cells,[70] necessary for lymphangiogenesis. Angiogenesis via VEGF-C and -D release can be mediated by binding to Flt-4 and KDR on blood vessel endothelium.

activates Flt-4 but not KDR, induced growth of lymphatic vessels in the skin of transgenic mice.[39]

Next to specific lymphatic growth factors VEGF-C and VEGF-D (figure 1), other factors may play a role in tumor-induced lymphangiogenesis. Due to a lack of detailed studies, the origin of lymphatic endothelial cells in adult angiogenesis is still unknown. It is likely that lymphatic endothelial cells originate from pre-existent lymphatics or from blood vasculature present at the tumor site. The gradual limitation of Flt-4 expression to only the lymphatic endothelial cells during late embryogenesis [14] and the induction of Flt-4 expression on angiogenic tumor blood vessels [18,19] support the generally accepted assumption that lymphatics are derived from blood vessels and in particular, from veins.[70] Similar to the formation of blood vessels during embryogenesis, circulating (bone marrow-derived) precursor cells in the blood or lymphatic stream could also participate in the formation of new lymphatics.

The role of Flt-4 expression in tumor-induced angiogenesis

During mouse embryogenesis, Flt-4 expression starts at embryonic day (E) 8 in developing blood vessels, but becomes largely restricted to the lymphatic endothelium after their formation from day E 12.5 onward.[14,57] The essential involvement of Flt-4 in blood vessel formation during embryogenesis is further emphasized by a study which demonstrated that targeted disruption of Flt-4 in mouse embryos prevented the remodeling of the primary vascular network and caused death at day E 9.5.[71] Flt-4 expression is also present on tumoral blood vessels, suggesting participation of Flt-4 in tumor angiogenesis as well.[18,19,72,73] Indeed, blocking of Flt-4 function resulted in impaired or absent branching of secondary and tertiary vessels in xenografted tumors,[72] thereby indicating that Flt-4 may have a similar role in tumor-induced and embryogenetic angiogenesis: remodelling of the primitive plexus into a higher-ordered architecture. Angiogenesis mediated by Flt-4 signal transduction is likely to occur after binding of its known receptors VEGF-C and VEGF-D (figure 1). Initially, it was reported that VEGF-C is able to induce angiogenesis in adult life

in the mouse cornea, ischemic muscle and a xenograft model.[43,74,75] However, overexpression of a Flt-4-specific mutant of VEGF-C and VEGF-D induced the growth of lymphatic vessels in the skin of transgenic mice,[39] thereby indicating that VEGF-C and VEGF-D induced angiogenesis is mediated predominantly by KDR, rather than by Flt-4.

If Flt-4 expression on blood vessels is restricted to malignant tumors, it may have diagnostic, prognostic and even therapeutic implications. In case of histological diagnostic difficulties, immunohistochemical analysis of Flt-4 expression may provide additional support in determining whether a tumor is benign or malignant and, perhaps, what its grade of malignancy is. Anti-angiogenic therapy aimed at Flt-4 may selectively destroy tumor blood vessels and reduce angiogenesis, whereas the normal blood vasculature remains unaffected. Also, lymphatic spread may be inhibited by destruction of lymphatic vessels and preventing lymphangiogenesis.

Although there are clear indications for the function of Flt-4 expression on tumor blood vessels,[72] it is yet unknown how this reappearance of Flt-4 is mediated. It is likely that factors released by the tumor cells themselves induce Flt-4 expression in a paracrine manner since the expression is present on blood vessels which are directly adjacent to tumor cell nests.[19] So, identifying the key players involved in Flt-4 induction would be of significant importance for our understanding of the mechanism of tumor-induced angiogenesis.

Absence of lymphatic vasculature in uveal melanoma

Melanoma of the uvea is the most common primary malignant intraocular tumor in adults,[76] originating from neuroectodermally derived melanocytes in the iris, ciliary body or choroid (figure 2). The highly vascularised choroid is the largest part of the uveal tract and the most common site for intraocular melanoma (80%).[77] These posterior melanoma lesions disseminate exclusively by a hematogenous route to the liver,[78] whereas anterior (iris) uveal melanomas may also metastasize to local cervical lymph nodes after invasion of conjunctival lymphatics.[79,80] The pure hematogenous spread of posterior tumors is generally explained by the assumption that lymphatic channels are absent from the eye and its derived melanoma. As depicted in figure 1, VEGF-C, VEGF-D and Flt-4 are thought be involved in tumor-induced lymphangio-genesis. However, as stated above, other factors may play a

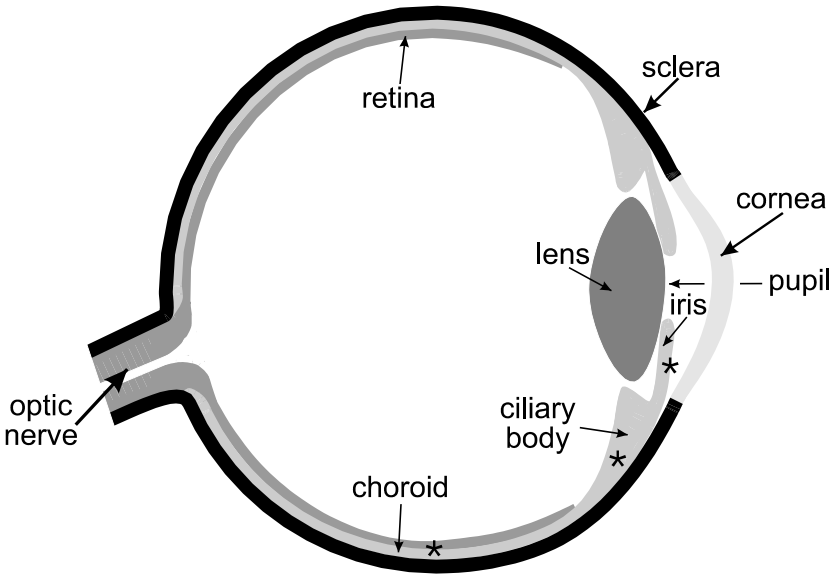

Figure 2. Schematic drawing of parasagital section of the human eye. The different layers of the bulbus oculi are depicted to show the localizations where primary uveal melanoma may arise (asteriks).

role in this process such as pre-existent blood and lymphatic vessels which may serve as a source of lymphatic endothelial cells. Evaluation of the lymphangiogenic proteins in a tumor type in which no lymphatic vessels are present (such as uveal melanoma) may therefore be useful in the identification of these additional factors.

Microvascular or extracellular matrix patterns in uveal melanoma

In the early nineties, Folberg et al. presented nine morphological types of microvessel architecture in uveal melanoma.[81] These microvascular patterns were identified by fluorescein-conjugated Ulex europaeus I as a marker for endothelium and by periodic acid-Schiff (PAS) histochemistry that stains basement membranes and collagen.[81,82] The patterns included: 1) the normal pattern (pre-existing blood vessels), 2) the silent pattern (lacking pre-existent tumor vessels), 3) the straight pattern (randomly oriented straight vessels that are not linked to one another), 4) the parallel pattern (straight vessels that are arranged to one another), 5) the parallel with cross-links pattern (vessels of parallel patterns that are also linked to each other), 6) the arcs patterns and 7) arcs with branching patterns (curves of vessels that form incomplete loops), 8) the loops pattern (vessel loops that are completely closed) and 9) the network pattern (composed of at least three back-to-back closed loops). By definition, if networks are present, loops are present.[81]

In uveal melanoma, the PAS-positive patterns arranged in arcs, loops and networks proved to be related to rate of metastasis and, hence, prognosis.[81,82] Since then, similar patterns in human metastatic cutaneous melanoma, breast, ovarian and prostate carcinoma were found and these could be related to prognosis as well.[83-87] These data indicate that the patterns play a biological role in tumor growth and metastasis. Their exact nature has become an extensive issue of debate in recent literature.[81,82,88-92] It was suggested that they were, in fact, blood vessel like vascular channels.[81,82] Re-assessment by an additional study questioned the true microvascular nature of the patterns [88] as Foss et al. documented that the patterns consisted of extracellular matrix in which blood vessels were embedded. Furthermore, it was suggested that 'vascular channels' were associated with the PAS-positive arcs, loops and network patterns.[93] Vascular channels are blood-conducting channels lined by tumor cells (and not by endothelium) in close contact with the blood vasculature. In these channels along the PAS-positive patterns, melanoma cells mimic endothelial cells (i.e. vasculogenic mimicry [90,93]). The finding of the possible existence of vasculogenic mimicry is of great significance for current insight in tumor biology and treatment: a tumor containing vascular channels would not be fully dependent on angiogenesis for growth and metastasis and it would be less sensitive to drugs aimed at interfering with endothelial cells.

Besides a possible role in tumor perfusion, the PAS-positive patterns may also be involved in tumor infiltration by inflammatory cells, since recent studies reported the presence of macrophages along the arcs, loops and network patterns.[94,95] In general, tumor-associated macrophages are present as a major component of the host leukocyte infiltrate and their presence is related to poor prognosis in several types of malignant tumors, including uveal melanoma.[94,96-98] These data point at an important role of macrophages in tumor growth and metastasis. Chemotactic cytokines provide the directional stimulus for the movement of leukocytes and are thought to be important in

the recruitment of monocytes into tumors. Several cytokines have been identified in this recruitment, including monocyte chemotactic protein-1, macrophage colony stimulating factor and VEGF-A (for extensive review: see ref. 99). In addition, endothelial-monocyte activating protein-II (EMAP-II) and VEGF-C have macrophage-chemotactic properties. EMAP-II was originally described as a tumor-derived cytokine, isolated from MethA tumor supernatant.[100,101] It is involved in embryonic development,[102-104] inflammation [105,106] and autoimmune disease.[107] Tumor-associated macrophages may play a role in tumor progression and regression. For example, it is known that macrophages promote tumor angiogenesis.[108] Furthermore, they may be involved in the genesis of the PAS-positive patterns.

Outline of this thesis

Despite the significant role of blood vessels, lymphatics and the arcs, loops and network PAS-positive patterns for tumor growth, progression and metastasis, especially the role of lymphatics, the presence of tumor-induced lymphangiogenesis and the nature of the PAS-positive patterns require further attention. Therefore, we decided to study the following issues:

1. To investigate the anatomical relationship between the lymphatic vasculature and tumor cells, a reliable immunohistochemical detection of lymphatics is required. Therefore, in **chapter 2** we further explored the differential blood and lymphatic staining by the previously described PAL-E/CD31 double-staining protocol [9] in a panel of normal and malignant tissues. In addition, Flt-4 expression on blood vessels as a possible progression marker was analyzed in a series of human melanocytic skin lesions with increasing malignancy.
2. For a better understanding of tumor-induced angiogenesis, identification of tumor derived factors that are involved in the induction of Flt-4 expression on blood vessels, would be useful. In **chapter 3**, we addressed the role of VEGF-A in this process using a xenograft mouse model of hematogenous human melanoma brain metastasis.
3. In addition to VEGF-C and VEGF-D, other factors are involved in tumor-induced lymphangiogenesis. In **chapter 4**, we determined the absence of lymphatics in primary uveal melanoma and studied the role of VEGF-A, VEGF-C, KDR and Flt-4 in this tumor type.
4. Since the nature of the PAS-positive patterns is essentially unknown, we analyzed morphological and functional properties of these patterns in primary uveal and xenografted melanoma in **chapters 5** and **6**.
5. In **chapter 7**, we further extended our study of the role of the PAS-positive arcs, loops and networks patterns by investigating the role of EMAP-II and VEGF-C in chemotaxis of monocytes and macrophages in primary uveal melanoma.

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2 Induction of Flt-4 expression on tumor microvasculature as a new progression marker in human cutaneous melanoma

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SUMMARY

The anatomical relation between a malignant tumor and its vascular and lymphatic bed is an important factor influencing metastasis. Lack of specific markers for the lymphatic endothelium has long hampered a reliable detection of lymphatics. Here, we demonstrate that lymphatic endothelium can reliably be identified in a panel of different normal tissues and of benign and malignant tumors. Application of the previously described PAL-E/CD31 double staining protocol differentiates between blood capillaries and veins on one hand and lymphatic vessels on the other. Blood vessel marker CD34, absent from lymphatics, was used additionally to classify arteries. We found that the lymphatic Flt-4 receptor (also known as vascular endothelial growth factor receptor-3 (VEGFR-3)) was present on both lymphatic and blood vessels in 76 of 113 malignant tumors (adenocarcinoma of kidney (n=3), colon (n=3) and liver (n=3), breast (n=9) and squamous cell carcinoma (n=5), primary (n=81) and metastatic (n=9) melanoma). No evident signs of tumor-induced lymphangiogenesis were observed. Evaluation of a series of 110 melanocytic skin lesions indicated that Flt-4 expression is confined to the lymphatic vasculature in benign lesions. However, its expression emerges on the blood neovasculature in malignant lesions as soon as metastatic potential develops. We conclude that induction of Flt-4 expression on tumor blood vessels may be a general phenomenon which would make Flt-4 a marker for tumor endothelium. In addition, we propose Flt-4 expression as a new microvascular progression marker in cutaneous melanoma.

INTRODUCTION

The dependence of tumors on the presence of a vascular bed for outgrowth and metastasis has now been firmly established.[1,2] Although tumor cell invasion of lymphatics worsens prognosis significantly,[3,4] it is still unknown why several tumors have a tendency to spread via the lymphatic bed. Evaluation of the anatomical relation between tumor mass and lymphatic bed has not been studied in much detail due to the lack of specific lymphatic markers.[5] Until recently, it was generally assumed that intratumorally no lymphatic vessels are present and that tumors are not able to induce formation of new lymphatic vasculature (lymphangiogenesis).[6] This is confirmed in a number of cases of primary cutaneous melanoma lesions and in a murine sarcoma model.[7,8] However, the possibility that lymphangiogenesis may occur in human malignant tumors is suggested by several animal studies demonstrating that xenografted tumors induced lymphangiogenesis as a result of overexpression of vascular endothelial growth factor-C (VEGF-C) or –D.[9-13]

More recently, several different specific lymphatic endothelial markers have been described which are not yet widely available however.[14-17] Therefore, blood and lymphatic vessels have been differentiated often by exploiting histo-morphological differences.[18-20] In a previous study,[7] we developed a staining protocol (using the pan-endothelial marker CD31 and the blood vessel endothelial marker PAL-E) that differentially highlights blood and lymphatic vasculature in normal skin and primary melanoma lesions. Arteries are not decorated by this staining protocol but this problem was overcome by supplementing the staining protocol with a monoclonal antibody (mAb) to the endothelial marker CD34.[21] Staining for CD34 of the endothelia of arteries, venules and capillaries has been demonstrated in normal and tumor tissues.[18-20] The issue whether CD34 is expressed on lymphatic vessels is not completely settled since its absence [15,18] but also a variable, presumed lymphatic staining have been described.[19,20,22]

Another, direct way of identification of lymphatics was more recently reported using the 9D9 mAb.[23] 9D9 specifically recognizes the Flt-4 receptor (also known as vascular endothelial growth factor receptor-3 (VEGFR-3)) that is expressed on lymphatic endothelium in normal adult tissues.[24] However, the use of Flt-4 as a marker for lymphatic vessels in human disease is dubious since Flt-4 expression on blood vessels has been reported in different types of malignant tumors and granulation tissue.[21,25-27] Flt-4 ligands are VEGF-C and -D,[28-30] of which VEGF-C induces both blood and lymphatic vessel development.[9-12,31-33] So, the presence of Flt-4 on tumor vessels together with VEGF-C and/or -D might be indicative for both hem- and lymphangiogenesis in human cancer.

Recent data suggest that Flt-4 expression on blood vessels may be a general phenomenon in human cancer.[21,25,26] Furthermore, if this expression does not occur in normal tissues, it may have biological and clinical implications. Theoretically, it may be related to the rate of malignancy, and therapeutical interventions using Flt-4 as a target might selectively reduce tumor growth by inhibition of both hemangiogenesis and lymphangiogenesis with possibly little side effects. In human skin, proliferation of melanocytes can give rise to common acquired melanocytic nevus, atypical (dysplastic) nevus, melanoma in situ, primary melanoma and melanoma metastasis (referred to as "stages of

melanoma progression"). The probability of metastasis considerably rises once the stage of a thick (lesion thickness > 1.5 mm) primary melanoma lesion has been reached. In the present study, this series of melanocytic lesions was taken as a model to evaluate whether Flt-4 expression on tumor blood vessels may serve as a tumor progression marker.

Thus, we evaluated blood and lymphatic vessel staining by the PAL-E/CD31 staining protocol, and by the QBEnd/10 (anti-CD34) and 9D9 (anti-Flt-4) [23,24] mAbs in a panel of different human normal and tumor tissues. The analysis focused on the following questions: 1) can blood and lymphatics be identified using serial sections stained by PAL-E/CD31 and CD34 in these tissues, 2) is Flt-4 expressed on blood vessels in the included tumor types, 3) are intratumoral lymphatics present in the various tumor types and can signs of lymphangiogenesis be observed, and 4) is Flt-4 expression on blood vessels a tumor progression marker?

MATERIALS AND METHODS

Patient Material

Frozen specimens of 20 normal and 52 tumor human tissues (Table I) were obtained from the pathology archives of the University Hospital Nijmegen where they were stored at -130^0C. Additional primary melanoma lesions (n=10) were kindly provided by Dr. U. Hofmann (Universitäts-Hautklinik, Würzburg, Germany). 68 paraffin-embedded primary cutaneous melanoma lesions (36 tumors with a Breslow diameter of < 0.75 mm; 17 tumors between 0.76-1.50 mm; 7 tumors between 1.50-3.00 mm and 8 tumors of > 3.00 mm) and 3 paraffin-embedded squamous cell carcinomas of the larynx were obtained from our pathology archives. All specimens had been diagnosed by a pathologist.

Table I. Tissues included in this study*

normal tissues	tumor tissues
breast (n=3)	breast cancer (invasive intraductal carcinoma, n=8; invasive lobular carcinoma, n=1)
colon (n=3)	colon (adenocarcinoma, n=3)
kidney (n=3)	kidney (adenocarcinoma, n=3)
liver (n=3)	liver (hepatocellular carcinoma, n=3)
preputial skin (n=5)	larynx (squamous cell carcinoma, n=5)
skin (n=3)	skin (common acquired melanocytic nevus, n=10) **
	skin (atypical (dysplastic) nevus, n=10) **
	skin (melanoma in situ, n=7) **
	skin (superficial spreading melanoma lesions, n=60) **
	skin (nodular melanoma lesions, n=11) **
	skin (acrolentiginous melanoma lesions, n=3) **
	skin (melanoma metastasis lesions, n=9) **

* in total 20 normal and 133 tumor tissues were included

** stages of melanoma progression

Antibodies

The mAbs used for immunohistochemistry were the endothelial markers CD34 (QBEnd/10) (DAKO, Glostrup, Denmark) and Flt-4 (9D9) (Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, Helsinki, Finland), the pan-endothelial marker CD31 (PECAM-1) (British Biotechnology,

ITK Diagnostics, Uithoorn, The Netherlands) and the blood vessel endothelial marker mAb PAL-E (our laboratory). Vascular staining by a batch of PAL-E hybridoma supernatant was optimized and evaluated in frozen skin sections. Polyclonal antibodies (PAbs) to Flt-4 (Santa Cruz, CA, USA) were purchased.

Immunohistochemistry

Except otherwise indicated, all incubation experiments were performed at room temperature. Four micron cryosections were air-dried and fixed in acetone at room temperature for 10 minutes. By haematoxylin and eosin (H&E) staining, representative sections of the included normal or tumor tissues (Table I) were identified. Areas in the normal tissue sections corresponding to tumor sites, were used as controls. Blocking of non-specific binding of biotin/avidin system reagents (Biotin/Avidin blocking kit, Vector Laboratories, Burlingame, CA, USA) was performed when necessary. After the first and all following incubation steps, the sections were rinsed with ample phosphate-buffered saline (PBS). Three successive sections of each specimen were incubated with 9D9, QBEnd/10 and PAL-E for 60 minutes, as previously described.[7] Then the secondary biotinylated affinity-purified anti-mouse IgG (Vectastain, Vector Laboraties) was incubated for 30 minutes, followed by a 45-minute incubation for the PAL-E and CD34 stainings and 30 minutes for the 9D9 staining with peroxidase-labelled biotin avidin complex (Vectastain). Subsequently, the 9D9 sections were incubated for 5 minutes with biotinylated tyramine, followed by a 20-minute incubation with the ABC-peroxidase solution (CARD method [34]). All three stainings were developed by a 10-minute incubation with a 0.4 mg/ml amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). In a part of the tumor tissues, a similar staining procedure was conducted with the PAbs to Flt-4. Sections prestained with PAL-E were incubated with the anti-CD31 antibody 60 minutes. The secondary alkaline phosphate-labelled rabbit-anti-mouse IgG was incubated for 30 minutes. The second staining was developed for 10 minutes with a mixture of 1 mg/ml Fast Blue, 0.2 mg/ml naphthol phosphate and 0.24 mg/ml levamisole (Sigma-Aldrich, Bornem, Belgium).

Four micron paraffin-embedded serial sections were deparaffinized in xylene and rehydrated in an ethanol series. Antigen-retrieval was performed by boiling the sections for 10 minutes in sodium citrate buffer (pH=6.0) using a microwave oven. Endogenous peroxidase activity was blocked by treating the sections for 30 minutes with PBS containing 3% hydrogen peroxide. Then the sections were incubated with 20% normal horse serum (Vector Laboraties) for 10 minutes. Two successive sections of each specimen were incubated with 9D9 mAb and QBEnd/10 mAb overnight at 4^0C and further processed as described above.

In control sections, primary antibodies were omitted. The following tissues were included as positive controls: normal skin, melanoma and breast carcinoma lesions in case of PECAM-1, PAL-E and 9D9 stainings [7,25,26,35] and hepatocellular carcinoma in case of QBEnd/10.[36-38] The 9D9 and QBEnd/10 stainings were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature. All sections were mounted in Imsol-mount medium (Klinipath B.V., Duiven, The Netherlands).

Determination of blood vessel density

In case of the primary melanoma lesions, we selected the superficial spreading melanoma lesions to evaluate the microvascular density (MVD) as a parameter of the extent of hemangiogenesis which is a measure for tumor progression. The nodular and acrolentiginous melanoma lesions were excluded to avoid biasing data obtained. By visual examination of the PAL-E/CD31 and CD34 immunostainings at 63x magnification, blood vessels in 10 nonoverlapping fields per tumor lesion were counted and averaged. Vascular counts included complete cross sections, partial cross sections and small groups of positive cells.

RESULTS

Evaluation of the PAL-E/CD31 and CD34 staining protocols in frozen sections

In all normal and tumor tissues (Table I), both immunostainings decorated vascular structures, which could be identified on the basis of their architecture and characteristic staining pattern, as described earlier (Table II,[7,18-20]) (figure 1a-d, appendix).

As previously shown,[7,21] the PAL-E/CD31-double staining differentially highlighted blood and lymphatic capillary beds in all normal (figure 1a) and tumor tissues (figure 1b). Because of PAL-E staining on arterial vessels was absent,[39] PAL-E negative and CD31 positive ($CD31^+/PAL\text{-}E^-$) ones were classified as lymphatic or arterial vessels whereas PAL-E and CD31 positive ($PAL\text{-}E^+$) ones were classified as blood vessels.[7,21] Staining of infiltrating leukocytes by CD31 was prominent as well (figure 1b).

Compared to the PAL-E/CD31 double staining, the anti-CD34 mAb stained $PAL\text{-}E^+$ blood vessels and $CD31^+/PAL\text{-}E^-$ vessels in all included tissues. Using the differences in morphological characteristics of arterial and lymphatic vessels, the $CD34^+$ and $CD31^+/PAL\text{-}E^-$ vessels could be classified as arteries. No staining of lymph vessels by the anti-CD34 mAb was observed in any of the normal tissues and tumor tissues (figure 1c,d, appendix). Also, stromal areas in sections of colon, colon carcinoma and (adnexal structures in) skin were positive using CD34 immunohistochemistry.

Table II. Summary of the endothelial staining patterns in frozen sections

		normal tissues:		tumor tissues	
antigen	**mAb**	**blood vessel**	**lymphatics**	**blood vessel**	**lymphatics**
CD31	PECAM-1	+*	+	+*	+
unknown	PAL-E	+	-	+	-
CD34	QBEnd/10	+	-	+	-
Flt-4	9D9	-	+	+**	+

+ = intensive staining (arrowhead, figure 1e), - = no staining (arrow, figure 1e)

* since PAL-E and anti-CD31 antibodies were used in a double staining protocol, it is not possible to identify CD31-staining of the capillary and venous blood vessels. Capillary and venous CD31 staining is overruled by the PAL-E staining 7 (figures 1-3)

** the occurrence of blood vessel staining in the various tumors by the anti-Flt-4 mAb is shown in more detail in Table III

Vascular Flt-4 expression in normal and tumor tissues on frozen sections

Despite extensive testing, immunostaining by the anti-Flt-4 PAbs in our hands resulted in a high background staining of especially stromal areas and unreliable, irreproducible staining of the vasculature. Therefore, we excluded these PAbs from further analysis.

Using serial PAL-E/CD31 and CD34 stainings, Flt-4 expression detected by the anti-Flt-4 mAb could be localized on lymphatic vessels in all normal tissues (figure 1e, Table II). No or very weak expression of Flt-4 on blood vessels was observed. However, we observed expression of Flt-4 on both blood and lymphatic vessels in 33 of the 42 investigated malignant tumors (Table II,III, figure 1b,f). In most malignant tumors, blood vessels surrounding nests of tumor cells were stained by the anti-Flt-4 mAb. In kidney and hepatocellular carcinoma, a more intense staining of the intratumoral blood vessels occurred compared to the other tumor types. In several malignant tumors within one section, both negative and positive Flt-4 staining of blood vessels was observed. The Flt-4 positive blood vessels were present in areas directly surrounding the tumor cells whereas Flt-4 negative ones were localized at further distance (figure 1f).

Table III. Incidence of Flt-4 expression on tumor blood vessels

tumor type	**positive cases***
breast (invasive intraductal and lobular carcinoma)	9/9
colon (adenocarcinoma)	2/3
larynx (squamous cell carcinoma)	4/5
kidney (adenocarcinoma)	3/3
liver (hepatocellular carcinoma)	3/3
skin (common acquired melanocytic nevus)	0/10
skin (atypical (dysplastic) nevus)	1/10
skin (melanoma in situ)	0/7
skin (superficial spreading melanoma lesions)	32/60
skin (nodular melanoma lesions)	11/11
skin (acrolentiginous melanoma lesions)	3/3
skin (melanoma metastasis lesions)	9/9

* Positivity was defined as staining (as shown in figure 1 and 3 (appendix)) by the mAb 9D9 (anti- Flt-4) of PAL-E^+ or $CD34^+$ vessels

Lymphatics and lymphangiogenesis in the tumor tissues

Presence of lymph vessels of kidney and liver carcinoma lesions were confined to the peritumoral areas. In common acquired melanocytic nevi, atypical (dysplastic) nevi, primary and metastatic melanoma, breast, colon and larynx carcinoma lesions, lymph vessels were also observed in the stroma between nests of melanocytes or tumor cells. These vessels may represent pre-existing lymphatics or may be newly formed (figure 1b). However, we were unable to observe evident signs of lymphangiogenesis, such as locally increased numbers of lymphatics or lymphatic sprouting. In addition, no major differences in localization or lymphatic density could be observed in the different stages of melanoma progression.

Flt-4 expression during stages of melanoma progression

The stages of melanoma progression are represented by the benign lesions common acquired melanocytic nevus, the premalignant lesions atypical (dysplastic) nevus and melanoma in situ, and the malignant lesions primary cutaneous melanoma (comprising superficial spreading, nodular and acrolentiginous melanoma) and melanoma metastasis (Table I). In these lesions, we analyzed vascular Flt-4 expression to assess its value as a progression marker. Sections of all 42 frozen melanoma lesions listed above, were evaluated. In addition, of the primary melanoma lesions, all 68 paraffin-embedded tumors were used. Lymphatic staining by the Flt-4 mAb was comparable between frozen and paraffin-embedded sections facilitating comparison of expression of Flt-4 by blood vessel endothelial cells in all specimens.

By comparison to a parallel PAL-E/CD31 double staining (frozen sections) or CD34 staining (paraffin-embedded sections), it became evident that Flt-4 expression in benign melanocytic lesions was strictly confined to lymphatics, whereas in 55 out of 83 malignant melanoma lesions it was present on both lymphatic and blood vessels (level of significance: $p<0.0001$, two-sided Fisher's exact test) (Table III, figure 2-3 (appendix), figure 4). In 2 benign lesions, Flt-4 was also found on dendritic Langerhans cells in the epidermis (figure 2a). In 2 superficial spreading melanoma lesions, extensive positivity of the epidermis, tumor cells and the epidermal area (i.e. blood and lymphatic endothelial cells, fibroblasts) was observed. Occasionally, dull Flt-4 staining of melanoma-associated macrophages was observed.

In case of primary melanoma lesions, we analyzed our data with respect to tumor thickness according to Breslow which is the most prominent prognostic marker in cutaneous melanoma.[40] As shown in Table III, none of the seven primary melanoma in situ lesions showed Flt-4 expression on dermal blood vessels whereas all 3 acrolentiginous melanoma (thickness between 0.7 and 3.2 mm, median 0.9 mm) expressed Flt-4 on their blood vessels. In case of 60 superficial spreading melanoma lesions, 28 lesions expressing no Flt-4 (thickness between 0.15 and 1.8 mm, median 0.60 mm) appeared to be thinner compared to 32 remaining lesions showing Flt-4 blood vessel expression

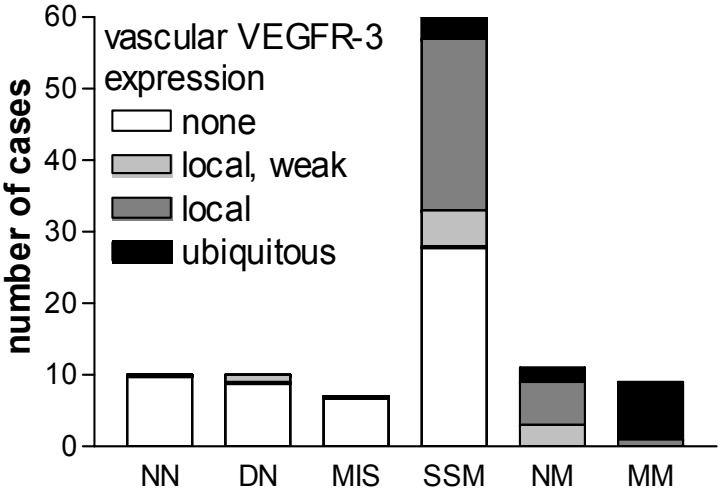

Figure 4. Bar diagram showing the distribution of vascular Flt-4 expression in the melanoma progression series which includes common acquired melanocytic nevi (MN), dysplastic nevi (DN), melanoma in situ (MIS), superficial spreading melanoma (SSM), nodular melanoma (NM) and metastatic melanoma (MM). Flt-4 blood vessel expression was classified as absent, locally and weakly present, locally present and ubiquitously present. For further details see text. Blood vessel Flt-4 expression was defined as staining (as shown in figure 2 and 3 (appendix)) by the mAb 9D9 (anti-Flt-4) of PAL-E^+ or $CD34^+$ vessels.

(thickness between 0.3 and 7 mm, median 1.2 mm) (p=0.0005, Mann-Whitney U test). In all 11 nodular melanoma (thickness between 2.1 and 11 mm, median 4.2 mm), Flt-4 was present on the blood vasculature.

In 40 of the 46 Flt-4 positive primary melanoma lesions, expression was present on a subset of blood vessels in restricted areas (often located at the invasive front of the tumor) whereas in the remaining 6 primary and 8 metastatic lesions Flt-4 expression was ubiquitous (figure 3a-d). No significant difference in thickness between the primary melanoma lesions with localized or ubiquitous Flt-4 expression was present.

Using routine haematoxylin and eosin staining, all 60 superficial spreading melanomas were classified as horizontal (HGP) or vertical growth phase (VGP) according to Elder et al.[41] and modified by Cook et al.[42] A higher number of VGP melanomas contained blood vessels expressing Flt-4 (24 out of 35 tumors) compared to the number of melanomas in HGP (7 out of 25) (p=0.008) (Fisher's exact test). Next to growth phase classification (i.e. HGP and VGP), which relates to the pattern of tumor invasion, we related vascular Flt-4 expression to the anatomical level of invasion, as defined by Clark et al.[43] As shown in figure 5, vascular Flt-4 expression is absent in non-invasive melanoma (Clark I) and the majority of minimal invasive tumors (Clark II) whereas vascular Flt-4 becomes present in the majority of tumors filling up the papillary or invading the reticular dermis (Clark III-IV) (p<0.0001) (Chi-square test).

Seven out of 17 most thin superficial spreading melanomas (thickness ≤ 0.5 mm) contained Flt-4-positive blood vessels. 5 of these positive melanomas were classified as VGP and the remaining 2 as HGP which both showed clear signs of regression (figure 3e,f). The remaining 10 thin Flt-4 negative melanomas were all classified as HGP. In addition, in 4 of the positive tumors, evident signs of immunological regression were present. In total, 7 out of 9 superficial spreading melanomas with evident signs of regression contained Flt-4-positive blood vessels.

The MVD in VGP lesions (mean density between 1.5-3.4 vessels per field, mean 2.1 vessels per field) was significantly increased compared with the HGP lesions (mean density between 0.85-2.4 vessels per field, mean 1.4 vessels per field) (Mann-Whitney U test, p=0.03), confirming earlier results [7] and indicating a process of hemangiogenesis. In tumors containing vascular Flt-4 expression, the MVD lesions (mean density between 1.6-3.4 vessels per field, mean 2.0 vessels per field) was also significantly increased compared with tumors without Flt-4 expression lesions (mean density between 0.85-2.7 vessels per field, mean 1.4 vessels per

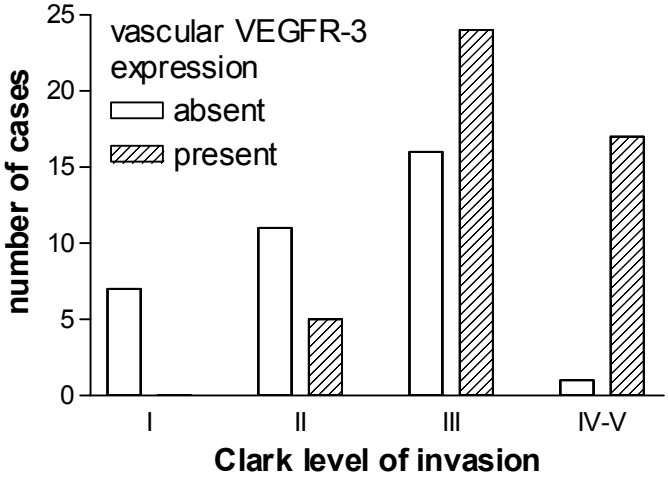

Figure 5. Bar diagram showing the distribution of vascular Flt-4 expression in relation to the anatomical level of invasion according to Clark et al.[43] Only one nodular melanoma lesion containing Flt-4 positive blood vessels invaded the sub-cutaneous tissue (Clark V).

field) (Mann-Whitney U test, p=0.02).

Data whether metastasis occurred were available in 57 (out of 74) primary superficial spreading, nodular and acrolentiginous melanoma lesions (follow up time: 4.1±2.2 years (mean±SD)). In this subset of 57 lesions, vascular Flt-4 expression was not related to the rate of metastasis (p=0.22, Fisher's exact test), although in 6 out of 7 metastatic lesions Flt-4 was expressed on tumor blood vessels.

DISCUSSION

In the past, it has been difficult to study lymphatic vasculature because of a lack of specific endothelial markers.[5] Here, we further extended the use of PAL-E/CD31 double staining in different normal and tumor tissues. Our results confirmed earlier studies [7,39,44] on endothelial specificity of these markers. Identification of lymphatics by the PAL-E/CD31 double staining protocol depends on the lack of lymphatic staining by the PAL-E mAb. Because of the absence of PAL-E staining on arterial vessels, antigen is present on all blood vessels with exception of arteries,[39,45] PAL-E negative vasculature could either be of lymphatic or arterial origin. Fortunately, these types of vasculature can easily be distinguished from each other by their morphological properties. The expression of CD31 by infiltrating cells may also hamper lymphatic detection in areas of extensive infiltration. So, with some limitations, the PAL-E/CD31 double staining is a reliable technique to classify vessel types. In agreement with previous studies,[15,18,20] CD34 was not detected on lymphatic channels in normal tissues. Although variable CD34 immunostaining of lymphatics was observed by others in a variety of vascular and lymphatic neoplasms,[19,20,22] this may be explained by their use of conventional histological criteria for vessel classification.[18-20] No direct comparison with a specific lymphatic staining was performed in these studies. We therefore suppose that these presumed lymphatics may have been, in fact, blood vessels. By combining the PAL-E/CD31 and CD34 stainings, lymphatics and blood vessels can reliably be detected in both normal and tumor tissues.

Flt-4 mRNA is detected on most endothelia in early embryogenesis, but later in development it becomes restricted almost exclusively to the lymphatic endothelium.[24] Recently, an elevated number of Flt-4 positive blood vessels was found in different tumor types.[21,25,26] By using the double staining protocol [7] that differentially highlights the lymphatic and blood vasculature, we have now confirmed these earlier results [21,25,26] in a panel of different tumor types including breast carcinoma, melanoma, squamous cell carcinoma of the larynx and adenocarcinoma of colon, kidney and liver. These earlier and our present results show that induction of blood vessel Flt-4 expression may be a general phenomenon in malignant human solid tumors which makes it a potential marker for tumor blood vessel endothelium. The involvement of Flt-4 in tumor-induced hemangiogenesis is supported by a murine model using transplanted tumors, showing that by blocking Flt-4 function this process is inhibited,[46] and by our study showing that vascular Flt-4 expression is associated with an increase of the MVD. Furthermore, its ligands VEGF-C and VEGF-D are able to induce both hemangiogenesis and lymphangiogenesis.[10-12,31-33] In this respect, it may be speculated that blood vessels expressing Flt-4 may be in an angiogenic state whereas blood vessels negative for Flt-4 are quiescent. This

hypothesis is supported by localisation of a subset of Flt-4 positive vessels at the invasive front of the tumor (this study) and by co-localisation of KDR (also known as VEGFR-2) (which may indicate endothelial activation [47]) and Flt-4 in uveal melanoma.[21]

Recent data provided by studies using transgenic mice which express soluble Flt-4,[48] have suggested that lymphangiogenesis occurs during adult life, confirming earlier findings (for review see: ref. 49). These mice do not develop lymphatic vessels but show some regeneration of these vessels during adult life. This latter finding not only shows that lymphangiogenesis occurs, but also indicates that receptors other than Flt-4 may be involved. Recent studies [9-12] have provided strong experimental evidence that tumors are capable of activating tumor lymphangiogenesis: overexpression of VEGF-C or –D by tumor cells in xenografts resulted in the development of an extensive lymphatic bed intra- and directly peritumorally. These studies indicate that VEGF-C mediated tumor-induced lymphangiogenesis may exist, which was recently confirmed in head and neck cancer patients.[50] In our study, in a number of lesions, lymphatics were predominantly present between tumor cells in the stromal areas. The limited number of the samples per tumor type studied did not allow for a clear distinction between pre-existent or tumor-induced lymphatic neovasculature. A higher lymphatic density in areas directly surrounding the tumor could not be determined. However, it is remarkable that we did not observe evident differences in localization and lymphatic vessel density during melanoma progression. This observation might suggest that the lymphatic vasculature may not be involved in the progression of benign melanocytic lesions to malignant melanoma. In addition, data of this study seems to confirm our previous finding that lymphangiogenesis does not occur in primary cutaneous melanoma.[7] However, in order to evaluate lymphangiogenesis in human cancer, comparison of lymphatic density to the normal tissue (in which the tumor arose) is absolutely necessary.[7] This comparison requires the presence of both normal and tumor tissue in one biopsy, which was also often not the case in our series.

Despite that our data strongly suggest that blood vessel Flt-4 expression is a melanoma progression marker, it remains questionable whether blood vessel Flt-4 expression is predictive of clinical and therapeutic outcome. Evident expression of Flt-4 by blood vessel endothelial cells was confined to the malignant lesions, i.e. those with dermal invasion and metastatic potential. In contrast, melanoma in situ which by definition does not invade the basal membrane and lacks metastatic potential, lacked Flt-4 expression. Moreover, in general, thin micro-invasive superficial spreading melanoma lesions (i.e. less than 0.60 mm) appeared to express no Flt-4 on their vessels whereas thicker superficial spreading and nodular melanomas did. The majority of thin superficial spreading melanoma lesions containing Flt-4-positive blood vessels showed signs of increased aggressiveness such as a vertical growth phase and regression.[51] Finally, the localized Flt-4 expression in primary melanoma lesions compared to the ubiquitous expression in melanoma metastatic lesions, also supports the hypothesis that Flt-4 expression gradually increases as a tumor becomes more malignant. So, following this hypothesis, evaluation of Flt-4 expression in melanoma lesions may be an interesting candidate for further clinical evaluation. For example, similar as recently suggested for the presence of VEGF in cutaneous melanoma,[52] detection of Flt-4 expression may

be helpful in those cases when differential diagnosis of benign and malignant lesions by conventional diagnostic methods (H&E staining), is difficult (which is often the case in the differential diagnosis of Spitz nevi and melanoma). Finally, the presence of blood vessel Flt-4 expression would have major therapeutic advances: by targeting Flt-4 expression, tumor vasculature may selectively be affected.

Recently, an association between a negative clinical outcome and expression of P- and E-selectin by endothelium of intratumoral vessels in cutaneous melanoma has been reported.[53] Together with our data, showing that primary and metastatic melanoma cells can cause Flt-4 expression on blood vessels in the skin and in distant tissues, these data point at an evident interaction of melanoma cells and their micro-environment as discussed previously.[54] In this respect, the question arises which factor induces Flt-4 expression on blood vessels. In breast carcinoma, blood vessels expressing Flt-4 were located adjacent to islets of tumor cells by which VEGF-C was expressed.[26] The fact that Flt-4 expression is restricted to the direct vicinity of tumor nests suggests that tumor-derived factors induce the appearance of Flt-4 in a paracrine way. Obviously, members of the VEGF-family are first target of investigation. So, thus far, most recent studies have assessed characteristics of melanoma cells themselves to predict clinical outcome. However, it is very likely that functional and morphological evaluation of the interaction of melanoma cells and surrounding stroma may yield data of substantial biological and clinical relevance as well.

In conclusion, for lymphatic staining the PAL-E/CD31 double staining combined with CD34 immunohistochemistry is well-suited. The presence of Flt-4 on tumor blood and lymphatic vessels is likely to play a role in mediating VEGF-C-induced hemangiogenesis and, perhaps, lymphangiogenesis. Our data suggest that induction of blood capillary and venous Flt-4 expression may be a general phenomenon in cutaneous melanoma and it may therefore be a marker for tumor endothelium. Finally, this induction may correlate with the degree of malignancy in human cutaneous melanoma.

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3 Expression of Flt-4 on tumor blood vessel endothelium is induced by VEGF-A_{165} in vivo via activation of KDR

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SUMMARY

In normal adults, Flt-4 (also known as vascular endothelial growth factor receptor-3 (VEGFR-3)) is restricted to the lymphatic vasculature. In malignant tumors, however, Flt-4 expression is induced on blood vessel endothelium. In a series of primary and metastatic cutaneous melanomas, we observed Flt-4 expression on blood vessels in close apposition to VEGF-A expressing tumor cells. To determine the role of VEGF-A, a human cutaneous melanoma cell line with low endogenous VEGF-A expression was engineered to express high levels of VEGF-A_{165}, or of a KDR (VEGFR-2) selective VEGF-A mutant. Immunohistochemical analysis of metastatic melanoma lesions in nude mouse brains showed that Flt-4 expression on blood vessel endothelium in these tumors is induced by VEGF-A_{165} via activation of KDR.

INTRODUCTION

Flt-4 (also known as vascular endothelial growth factor receptor-3 (VEGFR-3)) is essential for development and maintenance of the lymphatic vasculature.[1,2] During mouse embryogenesis, Flt-4 expression starts at embryonic day 8 in developing vessels, and becomes largely restricted to the lymphatic endothelium from day 12.5 onward into adulthood.[3] Overexpression of the Flt-4 ligands VEGF-C or -D (reviewed in ref. 4) in the skin of transgenic mice resulted in hyperplasia of the lymphatic vasculature (probably caused by lymphangiogenesis), leaving the blood vasculature unaffected.[1] Until now, the occurrence of lymphatic neovascularization in adult human tissue has only been reported in the context of wound healing.[5] However, overexpression of VEGF-C or -D by transfected tumor cell lines induced lymphangiogenesis in different mouse models,[6,7] opening up the possibility of lymphangiogenesis to occur in human cancer as well. In addition, VEGF-C was able to induce blood vessel angiogenesis (hemangiogenesis) in the cornea and malignant melanoma.[6,8] The presence of both VEGF-C receptors KDR (VEGFR-2) and Flt-4 on tumor blood vessels and of VEGF-C in different types of malignant tumors also suggested their involvement in tumor hemangiogenesis.[9,10] The role of KDR in tumor angiogenesis has been widely established (reviewed in ref. 4). In addition, a recent study [11] supported the additional involvement of Flt-4 in tumor hemangiogenesis by demonstrating that in xenografted tumors this process is inhibited by blocking Flt-4 function. Similar to VEGF-C, also VEGF-D may be involved in tumor-induced hemangiogenesis and lymphangiogenesis by binding and activating its receptors KDR and Flt-4,[6,7] respectively. The mechanism of Flt-4 induction on tumor blood vessels is unknown. In breast carcinoma, blood vessels expressing Flt-4 were located adjacent to islets of tumor cells by which VEGF-C was expressed.[12] The fact that Flt-4 expression is restricted to the direct vicinity of tumor nests suggests that tumor-derived factors induce the appearance of Flt-4 in a paracrine way. In a preliminary immunohistochemical study in primary cutaneous and metastatic melanoma, we observed co-localization of VEGF-A in tumor cells and Flt-4 expression on adjacent blood vessels. Together, these findings suggest that VEGF-A and VEGF-C both may be involved in the induction of Flt-4 expression on blood vessels. In the present study, we focused on the role of VEGF-A in the induction of Flt-4 expression on tumor blood vessels, by analysing expression patterns in primary and metastatic cutaneous melanoma. In addition, we examined Flt-4 expression in a mouse model of hematogenous brain metastasis utilising a human cutaneous melanoma cell line that was engineered to express VEGF-A_{165}. Finally, the effect of a KDR selective VEGF-A mutant was determined.

MATERIALS AND METHODS

Human melanoma lesions

Frozen specimens of 8 primary and 3 cutaneous metastatic skin and 2 metastatic lymph node lesions of cutaneous melanoma were obtained from the pathology archives of the University Medical Centre Nijmegen where they were stored at -130^0C. All specimens had been diagnosed by a pathologist.

Cell lines

The human melanoma cell line Mel57 with low endogenous VEGF-A expression was stably transfected with a plasmid pIRESneo (Clontech, Palo Alto, California) containing the coding sequence for VEGF-A_{165} (Mel57-VEGF-A_{165})[13] or a KDR selective VEGF-A variant. Generation and selectivity of the specific KDR-sel-2 VEGF-A mutant used have been described recently.[14,15] The transfected cells produced 300 ng recombinant protein/10^6 cells/24 hours while non-transfected cells produced less than 30 pg recombinant protein/10^6 cells/24 hours. The obtained cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Breda, The Netherlands).

Xenografts

Recently, we described a mouse model of brain parenchymal metastasis in which a modified internal carotid artery injection technique was used.[16] In BALB/c nu/nu mice, 10^5 cells of Mel57 (n=6), Mel57- VEGF-A_{165} (n=6) or Mel57-KDR-sel-2 (n=2) were injected into the right internal carotid artery. Mice were sacrificed in case of severe cachexia or acute development of neurological symptoms. All brains containing xenografted tumors were harvested and snap-frozen in liquid nitrogen.

Immunohistochemistry

Antibodies used for immunohistochemistry were polyclonal antibodies (PAbs) to mouse Flt-4 and human VEGF-A (Santa Cruz Biotechnology, Santa Cruz, USA), and monoclonal antibodies (mAbs) to human Flt-4 (9D9) (Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, Finland), mouse CD31 (Mec 7.46) (Hycult Biotechnology, Uden, The Netherlands), human CD31 (PECAM-1) (British Biotechnology, ITK Diagnostics, Uithoorn, The Netherlands) and PAL-E [17] (undiluted supernatant, our laboratory). Four micron cryosections were air-dried and fixed in acetone at room temperature for 10 minutes. Areas of interest were identified by routine H&E staining. Distribution of the binding of anti-VEGF-A, Flt-4 and CD31 antibodies was evaluated in serial sections using a standard three-step ABC method and development in 3-amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). All sections were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature. A double staining protocol using the pan-endothelial marker CD31 and the blood vessel marker PAL-E was performed as described recently.[10,18] The PAL-E/CD31 double staining differentially highlighted blood and lymphatic vasculature in human tissue. Finally, all sections were mounted in Imsol-mount medium (Klinipath B.V., Duiven, The Netherlands).

RESULTS

Primary and metastatic human melanoma

In all primary and metastatic lesions of cutaneous melanoma, the blood and lymphatic vasculature was differentially highlighted by the PAL-E/CD31 double staining as described previously (figure 1a, appendix).[10,18] By comparison with a serial PAL-E/CD31 double stained section, Flt-4 expression could be attributed to blood vessels in 6 out of 8 primary and all of 5 metastatic cutaneous melanomas (figure 1a,b). Occasionally, Flt-4 was also present on

subsets of melanoma cells (not shown). In the two primary melanomas with Flt-4 negative blood vessels also no or faint VEGF-A staining was detected (not shown). In the remaining 6 primary and all metastatic melanomas, VEGF-A expression varied considerably, both between and within the tumor nests. In tumor areas where VEGF-A expression was high, Flt-4 staining co-localised with a marked VEGF-A staining on endothelial cells (figure 1c,d). Occasionally, stromal and inflammatory cells were positive for VEGF-A. In tumor areas with low VEGF-A expression, endothelial cells were negative or, at most, weakly positive for Flt-4 (figure 1e,f).

Brain metastasis

To further evaluate whether VEGF-A release is able to induce Flt-4 expression on tumor blood vessels, we compared Flt-4 stainings in xenografts derived from the Mel57 parental and the transfected Mel57-VEGF-A_{165} human melanoma cell line. The absence of lymphatics from brain prevented a possibly confusing background of Flt-4 positive lymphatic vessels. The blood vasculature was identified by the use of an anti-CD31 mAb (figure 2a). The morphological and functional properties of the brain parenchymal vasculature induced by metastatic growth of Mel57 and Mel57-VEGF-A_{165} tumors have recently been investigated by us.[13] Untransfected Mel57 cells did not express VEGF-A and metastases formed by these cells appeared not to affect the cerebral vasculature. VEGF-A_{165} transfected cells, however, induced changes in the pre-existent vessels comparable to the "mother vessel" phenotype.[19] Flt-4 expression on blood vessels was absent in control brains without tumor (not shown) and in parenchymal blood vessels in unaffected brain areas in tumor-bearing mice. Serial section analysis showed that Flt-4 expression on tumor vessels in brain lesions of Mel57 was absent, both inside the metastatic lesions and peritumorally (figure 2b). In equally sized tumor islets in all Mel57-VEGF-A_{165} brain xenografts, however, Flt-4 expression was clearly induced, as a marked staining of the intra- and peritumoral vessels was observed. This Flt-4 expression on blood vessels gradually decreased with increasing distance from the tumor (figure 2c). Additional analysis of parental and VEGF-A_{165}-transfected Mel57 xenografts located in liver or peritoneum confirmed the induction of Flt-4 expression on blood vessels by VEGF-A_{165} (not shown). By mutating three amino-acids of the VEGF-A protein, the binding affinity of the resulting KDR-sel-2 VEGF-A mutant for Flt-1 (VEGFR-1) was almost completely lost. However, this KDR-sel-2 mutant appeared to bind and activate KDR.[14] Again, induction of Flt-4 expression on blood vessels by VEGF-A was observed in Mel57 lesions expressing this KDR-sel-2 variant (figure 2d).

DISCUSSION

In the present study, we demonstrated that Flt-4 expression on blood vessels is induced by VEGF-A_{165} in brain xenografts of human melanoma. Expression of a receptor-selective mutant showed that upregulation occurred via activation of KDR. The threshold levels of VEGF-A_{165} necessary for Flt-4 induction could not be determined, because we were not able to quantify the amounts of VEGF-A_{165} released by the xenografted tumor cells *in vivo*. We did show in an earlier study, however, that these cells produce high levels of VEGF-A *in vitro*.[13] As previously described,[13] VEGF-A_{165} over-expression by xenografted tumor cells

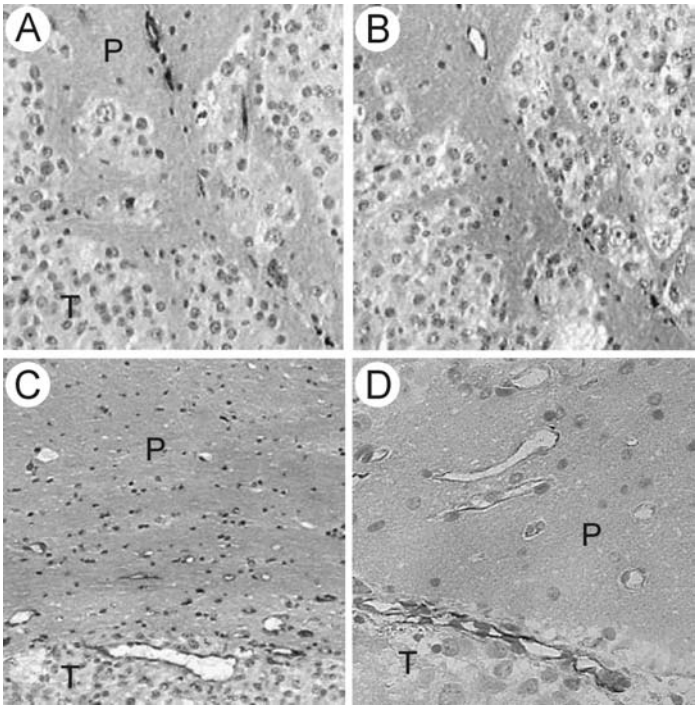

Figure 2. Immuno-histochemical analysis of the vasculature (using an anti-CD31 mAb, **a**) and Flt-4 expression (**b**,**c,d**) in brain melanoma metastasis in nude mice. In **a**,**c** and **d**, tumor (T) and brain parenchyma (P) are indicated. Flt-4 expression was completely absent in the normal brain (not shown), whereas serial section analysis showed no presence of Flt-4 expression on blood vessels in metastasis formed by the parental cell line (**b**). In metastasis formed by VEGF-A_{165} transfected melano-ma cells (**c**), blood vessel Flt-4 expression is clearly enhanced on both intra- and peritumoral vessels. Flt-4 expression on the vasculature decreases gradually with increasing distance form the tumor. In metastasis formed by KDR selective VEGF-A mutant transfected melanoma cells (**d**), Flt-4 expression is also present on the vascular endothelium. Counterstained with Harris' haematoxylin. Magnification **a**-**c**: 100x, **d**: 250x.

resulted in evident changes in tumor and blood vessel morphology. Therefore, we conclude from our present results that VEGF-A_{165} is released by the tumor cells, and that this factor is also responsible for the induction of Flt-4 expression on blood vessels in the brain metastasis model. Furthermore, signalling of VEGF-A via KDR is probably sufficient for this upregulation, since expression of the KDR-selective VEGF mutant led to a similar phenotype as seen after expression of wild type VEGF-A.

VEGF-A co-localised with Flt-4 on blood vessel endothelial cells in most of the human cutaneous melanoma examined. Conversely, in tumors with low VEGF-A levels, and also in stromal areas, VEGF-A and Flt-4 staining on blood vessels was absent. These findings are fully in agreement with our data from the murine brain metastasis model and support the hypothesis that VEGF-A upregulates Flt-4 expression on tumor blood vessels in human disease. In our animal model, this effect is mediated by binding to KDR. In an earlier study in primary uveal melanoma, we showed that KDR and Flt-4 are expressed on tumoral blood vessels in absence of VEGF-A but in presence of VEGF-C.[10] Since both VEGF-A and VEGF-C are ligands of KDR (for extensive review: see ref. 4) these data indicated that VEGF-C is also a possible mediator of Flt-4 induction in human cancer. Finally, the significance of VEGF-A binding to Flt-1 needs still to be evaluated.

Tumor lesions are surrounded by a peri-tumoral zone in which many interactions exist between stromal, inflammatory and pre-existent cells of the tissue in which the tumor arised. It becomes more evident that especially this peritumoral tissue is essential for outgrowth of tumors, as discussed recently.[20] The observed decreasing Flt-4 expression on blood vessels with increasing distance of the metastatic brain lesion (figure 2c) nicely illustrates the range

over which tumor-induced factors may influence the surrounding tissue, thereby demarcating the extent of the peri-tumoral zone.

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4 Lack of lymphangiogenesis despite co-expression of VEGF-C and its receptor Flt-4 in uveal melanoma

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SUMMARY

Because lymphatic vessels are absent from the normal eye and because uveal melanomas are presumed to spread by a hematogenous route in the absence of tumor exposure to conjunctival lymphatics, we investigated the presence of lymphatics in primary uveal melanomas. The presence of lymphatics in 2 control eyes, 33 primary uveal, 10 primary cutaneous and 3 metastatic cutaneous melanomas was evaluated by using a double immunostaining protocol that differentially highlights blood and lymphatic vasculature. In addition, 14 uveal melanomas were immunostained for the lymphatic growth factor vascular endothelial growth factor-C (VEGF-C) (anti-VEGF-C polyclonal antibodies (PAbs)), its receptors Flt-4 (monoclonal antibody (mAb 9D9)) and KDR (anti-KDR mAb (Clone KDR-2)) and for the hemangiogenic factor VEGF-A (anti-VEGF PAbs). Lymphatics were detected in normal eyes or in uveal melanoma. As a consequence, signs of lymphangiogenesis were not present. There was coexpression of VEGF-C with Flt-4 and KDR in 6 out of the 14 melanomas (43%). Staining for VEGF-A was completely negative in 25 uveal melanomas analyzed. The strictly hematogenous metastasis of primary uveal melanomas is explained by the absence of lymphatics in and around the tumor. The current data suggest that, in the presence of endothelial Flt-4, VEGF-C expression is not sufficient to induce lymphangiogenesis from preexisting blood vessels in human cancer.

INTRODUCTION

Primary uveal melanoma is the most common malignant intraocular tumor. It has a marked metastatic preference for the liver.[1] Once metastasis occurs, prognosis becomes very poor.[1] Microvessel density was identified as an important prognostic factor for many types of tumors.[2] However, in the case of uveal and cutaneous melanoma, there are conflicting reports on the presence of an association between microvascular density and prognosis.[3-7] In uveal melanoma, blood vessel architecture [3,8] [9] and both architecture and density [4] have been related to prognosis.

Dissemination of primary intraocular and posterior uveal melanoma occurs exclusively by a hematogenous route. Anterior uveal melanomas may also metastasize to local cervical lymph nodes after invasion of conjunctival lymphatics.[10,11] The restricted hematogenous metastasis is generally explained by the intraocular absence of lymphatics and the extrapolated assumption that, hence, this vessel type is therefore absent in uveal melanoma as well. However, this hypothesis has never been confirmed. In skin, lymphatic vessels are abundant, which explains the phenomenon of locoregional metastasis that is frequently observed in cutaneous melanoma. It is therefore possible that the mere presence of pre-existent lymphatics in skin contributes to the difference in metastatic pattern between uveal and cutaneous melanoma. Furthermore, the presence of lymphatics in cutaneous melanoma might obscure the relation between blood vessel density and prognosis that was established in uveal melanoma.[3,4] However, induction of lymphatic vessel formation (lymphangiogenesis) may play a role.

Angiogenesis is necessary for tumor growth.[12] In this process, the role of vascular endothelial growth factor (VEGF-A) has been firmly established. Another VEGF family member, VEGF-C, has been identified as a lymphatic endothelial growth factor.[13] VEGF-C expression has been observed in tumor cells,[14] which theoretically opens the possibility that tumors, besides hemangiogenesis,[15] also induce lymphangiogenesis.[16] During embryogenesis, lymphatics arise from venous endothelial cells, but whether veins can be the source of lymphatic neovascularization during adult life, should this occur, is unknown. Because VEGF-C exerts part of its function via the tyrosine kinase receptor Flt-4,[17] and because this receptor is upregulated in blood vessels of certain tumor types,[18,19] analysis of Flt-4 expression in uveal melanoma may be relevant as well. Should lymphatics in uveal melanomas be absent, VEGF-C expression in this type of tumor might influence hemangiogenesis, as reported in animal studies.[20,21]

To elucidate the nature of the vasculature in uveal melanoma and its role in mediating growth and metastasis, knowledge of the presence of a lymphatic vasculature becomes very relevant. In the present study, we have evaluated the presence of lymphatics in primary uveal melanoma and in primary and cutaneous metastatic lesions of cutaneous melanoma by using a double-immunostaining protocol (using the blood vessel endothelial marker PAL-E and the panendothelial marker CD31) that differentially highlights blood and lymphatic vasculature.[22] In addition, we studied the expression of VEGF-A, VEGF-C and the receptors KDR and Flt-4, the latter of which is believed to be specific for VEGF-C.

MATERIALS AND METHODS

Patient material

Frozen specimens of 33 primary uveal melanomas, 10 primary cutaneous melanomas, 3 cutaneous metastatic lesions of cutaneous melanoma, 2 uninvolved eyes, 1 invasive ductal breast carcinoma, 1 hemangioma and 1 normal preputial skin (Table I) were obtained from the pathology archives of the University Hospital Nijmegen where they were stored at -130^{0}C. Presence or absence of disease in all specimens had been determined by a pathologist. All primary melanomas and the uninvolved eyes were obtained by surgery. The uveal melanomas varied from 5 to 28 mm in diameter (median 17 mm) and included 31 choroidal and 2 ciliary melanoma lesions. Uveal melanoma lesions were divided in two parts along the maximal diameter. One part was formalin-fixed and the other part was snap-frozen. By using hematoxylin and eosin staining on paraffin sections, the uveal melanomas were classified as 11 spindle cell type and 22 epithelioid and mixed type. Azan staining without counterstaining on unbleached paraffin sections showed that 15 uveal melanomas contained the arcs, loops and networks matrix patterns (figure 1, i.e. PAS-positive patterns as described recently [8,23]).

Table I. Antibodies and positive controls used for immunohistochemistry

Antigen	Ab	Type of endothelium	Tissue
CD34	QBEnd/10	blood vessel	preputial skin, cutaneous melanoma
?	PAL-E	venous blood vessel and capillaries, not cerebral and retinal vessel*	preputial skin [25]
CD31	PECAM-1	blood and lymphatic vessel	preputial skin, cutaneous melanoma [22]
alpha-smooth muscle actin	α-SM1	-	preputial skin
KDR	Clone KDR-2	blood vessel	hemangioma [42]
Flt-4	9D9	lymphatic in normal tissues lymphatic and blood in tumor tissues	preputial skin, cutaneous melanoma [22]
VEGF-A	VEGF-A Abs	-	invasive ductal breast carcinoma [43]
VEGF-C	VEGF-C Abs	-	invasive ductal breast carcinoma [18]

* venous vessels that form a blood-brain barrier [30] or blood-retinal barrier [28]

Antibodies

Monoclonal antibodies used for immunohistochemistry (Table I) included anti-CD34 (QBEnd/10) (DAKO, Glostrup, Denmark), the blood vessel endothelial marker mAb PAL-E (undiluted supernatant, our laboratory), the pan-endothelial marker CD31 (PECAM-1) (British Biotechnology, ITK Diagnostics, Uithoorn, The Netherlands), anti-alpha-smooth muscle actin (α-SM1) (Sigma Chemical Co., St. Louis, USA), anti-KDR (Clone KDR-2) (Sigma, Saint Louis, Missouri, USA)

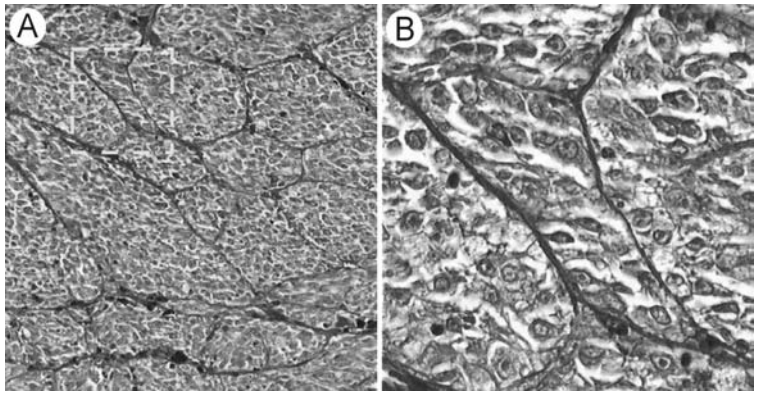

Figure 1. Evaluation of matrix patterns in a paraffin-embedded primary uveal melanoma stained by azan histochemistry. Extracellular matrix and nuclei of tumor cells were highlighted. Loops and network patterns were clearly visualized. The area indicated in **a** (magnification: 100x) is shown enlarged in **b** (magnification: 400x) demonstrating the clear decoration of the matrix patterns.

and anti-Flt-4 (9D9) (Molecular/Cancer Biology Laboratory, University of Helsinki, Finland). For VEGF-A and VEGF-C stainings, polyclonal antibodies (Santa Cruz, California, USA) were used.

Immunohistochemistry

Four-micrometer cryosections were air-dried and fixed in acetone at room temperature for 10 minutes. After incubation steps, sections were rinsed with ample phosphate-buffered saline (PBS). For the primary melanomas, each analysis included six successive sections of each specimen incubated with QBEnd/10 (diluted: 1:100), PAL-E (undiluted supernatant), anti-KDR (diluted 1:400), 9D9 (diluted: 1:1000) and the polyclonal antibodies to VEGF-A (diluted 1:20) and VEGF-C (diluted: 1:20) for 60 minutes at room temperature. Then, secondary 1:200 diluted biotinylated affinity-purified anti-mouse IgG (Vectastain, Vector Laboraties) (CD34, PAL-E, KDR and Flt-4) or affinity-purified anti-rabbit IgG (VEGF-A and VEGF-C) was incubated for 30 minutes, followed by a 45-minute incubation (CD34, PAL-E and VEGF-A) or 30-minute incubation (Flt-4, KDR, VEGF-C) with peroxidase-labeled biotin-avidin complex (Vectastain). Subsequently, the KDR, Flt-4 and VEGF-C sections were incubated for 10 minutes with biotinylated tyramine (dilution 1:200), followed by a 20-minute incubation with ABC-peroxidase solution (catalysed reporter deposition method [24]). All stainings were developed by a 10-minute incubation with 0.4 mg/ml 3-amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). For double staining, sections prestained with PAL-E were incubated with anti-CD31 antibody (diluted: 1:2000) for 60 minutes. The secondary 1:40 diluted rabbit-anti-mouse alkaline phosphate-labeled antibody was incubated for 30 minutes. The second staining was developed for 10 minutes with a mixture of 1 mg/ml Fast Blue, 0.2 mg/ml naphthol phosphate and 0.24 mg/ml levamisole (Sigma-Aldrich, Bornem, Belgium). In control sections, primary antibodies were omitted. Positive controls for all antibodies were included (Table I). The QBEnd/10, KDR, VEGF-A, VEGF-C and 9D9 stainings were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature. All sections were mounted in Imsol-mount medium (Klinipath B.V., Duiven, The Netherlands).

To validate our PAL-E/CD31 double staining protocol, we additionally stained serial sections of preputial skin by anti-CD34 mAb and by mouse anti-human alpha-smooth-muscle actin mAb (diluted 1:15,000). Furthermore, Masson trichrome histochemistry was performed as well, on an adjacent section.

By including positive controls, stainings of the normal and tumor tissues were validated. To exclude exogenous peroxidase activity or non-specific background, all serial stainings were incubated in a mixture of 1 ml 30% H_2O_2 in 200 ml acetone during fixation for 5 minutes and blocked by incubation with 20% normal horse (PAL-E, CD34, KDR, Flt-4, α-SM1) or 20% normal goat serum (VEGF-A and VEGF-C).

RESULTS

Vascular staining in human skin

To validate vascular staining protocols, preputial skin was used because of its richness in lymphatics. As described previously,[22] the PAL-E/CD31-double staining design was based on the reactivity of anti-CD31 mAb with both lymphatic and blood vessel endothelial cells, in combination with the reactivity of PAL-E with blood vessel endothelium alone. The blood vessel endothelial staining produced initially by anti-CD31 is overruled by staining by PAL-E. Thus, the vasculature was differentially highlighted in preputial skin sections (figure 2a, appendix). We found strong PAL-E positivity of blood capillaries and venules, but no staining of arterial vessels. The CD31 antibody staining, that was not masked by PAL-E, decorated both fine, thin-walled capillaries and arterial vessels. PAL-E and CD31 positive (PAL-E^+) vessels were classified as blood vessels and PAL-E negative and CD31 positive ($CD31^+$/PAL-E^-) ones were classified as lymphatic (or arterial) vessels.[22,25,26] The blood vessel specificity of the differential staining was confirmed by the presence of smooth muscle cells (figure 2a,b). Masson trichrome histochemistry, highlighting elastic fibers in blood vessel walls, confirmed this specificity (figure 2d). Because arteries and lymphatics are both negative for PAL-E, it is not always possible to differentiate these vessel types. As we have recently demonstrated,[27] an anti-CD34 mAb stained the PAL-E^+ blood vasculature and $CD31^+$/PAL-E^- arteries, whereas the $CD31^+$/PAL-E^- lymphatic vessels were negative for CD34 (figure 2a,c). Using this combination of stainings all vessels could be classified.

Vascular staining in normal eyes

Frozen specimens of the ocular wall of 2 normal eyes were evaluated. In the choroid layer, PAL-E^+ blood capillaries and venules were observed (figure 3a, appendix). As demonstrated previously,[28] no PAL-E positivity of the vasculature in the retinal layer containing the blood-retinal barrier [29] was observed. Since all $CD31^+$/PAL-E^- vessels were stained by the anti-CD34 mAb (figure 3b), these were identified as arterial vessels. No $CD31^+$/PAL-E^- lymphatics were observed in the choroid layer.

Vascular staining in cutaneous and uveal melanomas

In all primary uveal (n=33), cutaneous melanomas (n=10) and cutaneous metastatic lesions of cutaneous melanomas (n=3), PAL-E^+ blood capillaries and venules were observed, whereas $CD31^+$/PAL-E^- arteries were present in only 3 uveal melanoma lesions. In the primary and metastatic cutaneous melanomas, $CD31^+$/PAL-E^- lymphatics were observed in the pre-existent skin directly surrounding the tumor (figure 4a, appendix) and delicate lymphatics between tumor fields as described in more detail previously [22]. In uveal melanomas, all PAL-E^+ blood capillaries and venules and $CD31^+$/PAL-E^- arteries were also

stained by the anti-CD34 mAb. In none of the uveal melanomas, $CD31^+/PAL\text{-}E^-$ lymphatics were observed. A representative example is shown in figure 4b and c. Vascular staining by the PAL-E mAb was superior to staining by the anti-CD34 mAb (figure 4c,d). No evident staining of uveal melanoma cells was observed by any of the endothelial markers.

VEGF-A, VEGF-C, KDR and Flt-4 staining in uveal melanomas

Staining for VEGF-A was completely negative in series of 25 uveal melanomas. In total, 14 uveal melanomas were evaluated for VEGF-C, KDR and Flt-4 expression. Table II summarizes all staining results. 8 tumors were positive for expression of VEGF-C (figure 5a) and 6 tumors were negative. In the positive tumors, distinct areas of positive cytoplasmatic staining for VEGF-C were observed in the tumor cells directly surrounding Flt-4 positive blood vessels (figure 5a,b). VEGF-C expression colocalized with blood vessel endothelial Flt-4 expression in 7 tumors and in 6 of those with expression of endothelial Flt-4 and KDR (figure 5), whereas in only one VEGF-C positive tumor, neither Flt-4, nor KDR expression could be detected. (melanoma 2, Table II). In 9 tumors, endothelial Flt-4 expression was observed, and in 2 of those, no evident VEGF-C staining could be detected (melanoma 1 and 4, Table II). In 1 of these 2 latter tumors KDR was co-expressed (melanoma 1, Table II). In one tumor sample, two separate nodules were present, in one of which both VEGF-C, KDR and Flt-4 expression was observed, whereas in the other nodule these stainings were negative (melanoma 13, Table II).

DISCUSSION

To address the hypothesis whether the absence of lymphatic spread in primary uveal melanoma can be explained by the absence of lymphatic vasculature, we evaluated the presence of lymphatics and the expression of VEGF-C and Flt-4 in this type of tumor. Our staining approach made it possible to differentiate between lymphatic and blood vasculature, confirming our recent analysis in a variety of normal tissues and carcinoma types [22] and was validated in the present study by additional alpha-smooth-muscle immunostaining and Masson trichrome histochemistry. However, it is known that PAL-E is present on capillary and venous endothelium throughout the body [25] with the exception of vessels in areas in the brain with an intact blood-brain barrier.[30] In the eye, a similar barrier (i.e., blood-retinal), and endothelial PAL-E antigen expression is absent as well.[28] In areas in both brain and the eye where such a barrier is not present, the PAL-E antigen is expressed on the endothelium.[28,31] Therefore, it was not possible to evaluate blood and lymphatic staining by the PAL-E/CD31 double staining protocol in normal eyes alone. Because anti-CD34 mAb detects all types of vessel endothelium with exception of the lymphatic vasculature.[27,32] blue ($CD31^+/PAL\text{-}E^-$) vessels could be classified as blood or lymphatic vessels by comparison with CD34 expression. In uveal melanomas, all vessels were stained by PAL-E or CD34, confirming earlier results.[28,31] So, in conclusion, in this way, we were able to classify both types of vessels in preputial skin, normal eye and uveal melanoma sections.

In our specimens, the extent of melanin pigmentation did not interfere with the detection of the vascular patterns, making bleaching unnecessary. Azan histochemistry resulted in deep blue staining of extracellular matrix and red

staining of the cell nuclei whereas PAS-histochemistry is less powerful in extracellular matrix component detection. Indeed, for this approach, bleaching of melanoma sections and the use of a green filter during microscopy are required for reliable evaluation. However, in case of the presence of strongly pigmented melanophages along the matrix patterns (as seen in figure 4), bleaching may still be necessary differentiating between melanophages and the Azan-positive blue matrix patterns. The use of Azan histochemistry in identifying matrix patterns was confirmed by immunofluorescence and electron microscopy (data not shown).

Table II Overview of the expression of VEGF-C, Flt-4, KDR and the presence loops and networks pattern in 14 uveal melanoma

melanoma	VEGF-C	Flt-4	KDR	loops and networks
1	-*	+	+	-
2	+†	-	-	-
3	-	-	-	-
4	-	+	-	-
5	-	-	-	-
6	-	-	-	+
7	+	++	+	-
8	++‡	++	+	-
9	+	+	-	-
10	+	++	++	+
11	++	++	++	-
12	+	++	+	-
13	+	+	+	+
14	-	-	-	+
mean:	57%	64%	50%	
95% CI§:	28%-83%	35%-88%	23%-77%	

* -: expression or loops and networks absent
† + moderate expression or presence of loops and networks
‡ ++ strong expression (as depicted in figure 5)
§ CI: confidence interval

Electron microscopy has suggested that endothelium-free channels are lined by basement membrane.[33] Although PAL-E detects the epidermal basal membrane [22,34] this is a specific pattern and staining of basal membranes of endothelium-free channels in the fibrovascular patterns is not likely to occur. However, at

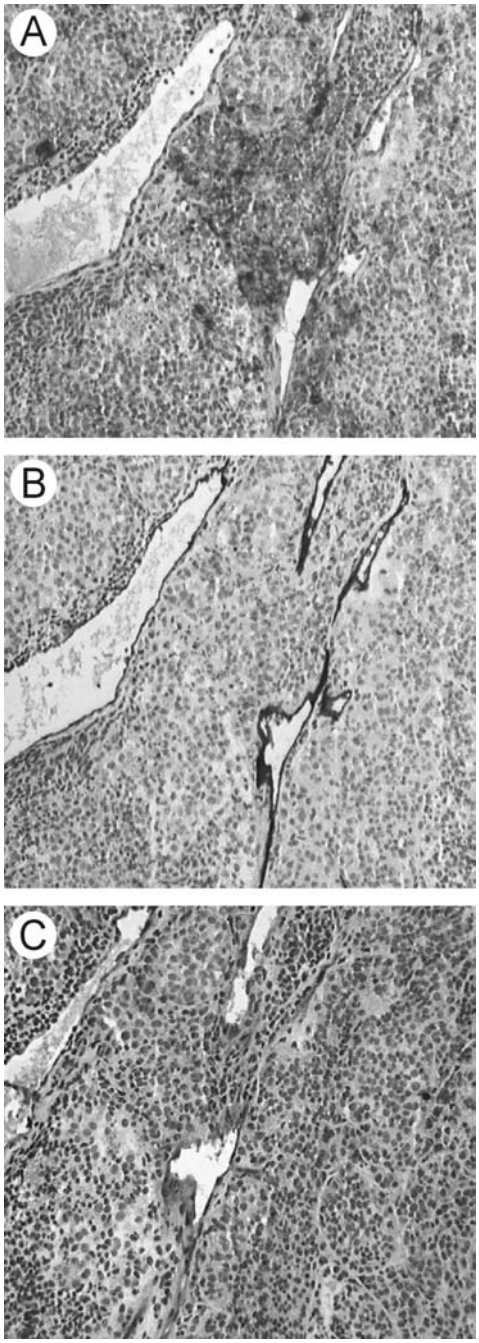

Figure 5. Immunohistochemical analysis of VEGF-C (**a**), Flt-4 (**b**) and KDR (**c**) expression in uveal melanoma. VEGF-C expression is located in part of the tumor cells surrounding the Flt-4 and KDR positive blood vasculature. Counterstained with Harris' haematoxylin. Magnification: 200x.

low magnification, the PAL-E/CD31 double staining may have given the impression that next to blood vessels, also certain parts of fibrovascular patterns were identified. Evaluation at higher magnifications (figure 4c and d) demonstrated, however, that this impression was caused by the presence of numerous melanin-laden macrophages spread along the vasculature. Furthermore, vascular detection by PAL-E mAb was superior to anti-CD34 mAb in all uveal melanomas, and in those melanoma lesions that did not contain arcs, loops and network patterns. In conclusion, the PAL-E/CD31 double staining protocol could not be used to evaluate the existence of endothelium-free channels.

Knowledge of the absence of lymphatics contributes to the evaluation of the existence and nature of the non-endothelialized blood conducting channels.[33] Although these are still controversial in uveal melanoma, the absence of lymphatics rules out the possibility that these channels were, in fact, lymphatic channels.

The present study demonstrates that lymphatics are absent from both the normal eye and uveal melanoma. This is in line with the lack of lymphogenous metastasis of intraocular and posterior uveal melanoma leaving only the hematogenous route open for dissemination. Although prognosis is related to microvascular density in uveal melanoma,[3,4] this correlation is absent in cutaneous melanoma.[35,36] In addition to blood vessels, lymphatics are involved in indirect metastasis to the blood stream. So, the absence of lymphatics in uveal melanoma and presence of lymphatics in cutaneous melanoma makes it plausible that lymphatics also play a role in determining the rate of distant metastasis and prognosis. In this respect, it is an interesting question whether lymphatic vessel density, or rather a combination, with blood vessel density is related to prognosis and metastatic spread in cutaneous melanomas. However, since we were not able to evaluate microvascular density in relation to prognosis to our series, we can not confirm this hypothesis.

Although blood vessel angiogenesis is an established phenomenon, it is unknown whether lymphangiogenesis occurs in human cancer. Because we can

not be absolutely sure that immature lymphatic vessels express lymphatic markers like CD31 or Flt-4, we can not rule out the occurrence of lymphangiogenesis in uveal melanoma. However, it is likely that life-span of larger tumors would allow maturation of lymphatic vessels after lymphangiogenesis. These mature vessels would readily be detected by our staining protocol. Furthermore, Flt-4 expression is present on sprouting lymphatic vessels during wound healing in the adult whereas lymphatic vessels remain PAL-E negative during development.[37] In our series, we did not observe such Flt-4^+ and PAL-E^- vessels. Thus, our study strongly suggests that lymphangiogenesis does not occur in this type of tumor although we can not rule out this completely.

VEGF-C has been identified as a lymphatic endothelial growth factor during embryogenesis [13,38]. In the present study, although VEGF-C was expressed and Flt-4^+ positive blood vessels are present concurrently as a source of endothelial cells (for review ref. 39), lymphangiogenesis did not occur in uveal melanomas. VEGF-C is, however, also able to induce hemangiogenesis.[20,21] In addition, its receptors KDR and Flt-4 are involved in angiogenesis (for review see ref. 40). Therefore, the clear relation between the expression of VEGF-C, KDR and Flt-4 suggests that the presence of VEGF-C and its receptors in uveal melanoma may contribute to hemangiogenesis. KDR is early involved in angiogenesis during embryogenesis and its expression becomes upregulated on tumor endothelium under hypoxia (for review see ref. 40) These data suggest that in tumor areas with local KDR expression, endothelium is in a state of angiogenesis whereas in areas lacking KDR, no new vessels can be formed. Therefore the coexpression of KDR and VEGF-C supports the role of VEGF-C as a hemangiogenic growth factor.

Many different types of tumors express VEGF-A, an important regulator of angiogenesis, indicating that tumor cells in addition to endothelial cells contribute to the tumor blood vasculature. Therefore, it was surprising that VEGF-A was absent from all uveal melanoma lesions, as demonstrated previously.[41] Because of the absence of VEGF-A, it appears that hemangiogenesis in uveal melanoma may instead be driven by VEGF-C. In this respect, the presence of VEGF-B and VEGF-D needs to be further evaluated.

In conclusion, although the lymphatic endothelial growth factor VEGF-C and its receptor Flt-4 are expressed, neither lymphatics, nor signs of lymphangiogenesis are present in normal eye and primary uveal melanomas, indicating that the concerted action of these players is not sufficient for lymphangiogenesis to occur in the adult in this type of tumor. Furthermore, hemangiogenesis in uveal melanoma is not associated with expression of VEGF-A, but may be driven by other angiogenic factors like VEGF-C.

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5 Presence of a fluid-conducting meshwork in xenografted cutaneous and primary human uveal melanoma

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SUMMARY

Recently, it was reported that tumor cells themselves generate channels and networks in three-dimensional culture and can be found lining channels (some containing red blood cells (RBCs)) in vivo, and they express endothelial or vascular genes in aggressive uveal melanoma. The implications of these data for current insights in the involvement of angiogenesis in tumor growth, metastasis and therapeutic intervention are considerable. Therefore, we evaluated this issue in our material. 30 human uveal melanomas and 20 xenografts of human cutaneous melanoma were analysed by Azan histochemistry and immunostaining of endothelial markers. Additionally, in xenografted tumors a tracer study using confocal microscopy and (immuno)-electron microscopy was performed. Lumina or spaces without endothelial lining containing RBCs were not detected in any lesion. Functional evaluation of the vasculature in xenografts demonstrated rapid tracer appearance both in- and outside blood vessels. Outside blood vessels it spread along matrix networks of arcs and back-to-back loops. Confocal microscopy showed that this extracellular matrix was deposited as stromal sheets around nests of tumor cells. Laminin immunostaining revealed that between sheets surrounding adjacent nests, spaces were present. These spaces were, however, filled with collagen and different types of cells, including cells stained for macrophage markers. Although no evident endothelium-free and RBC-containing channels were present in the tissues examined, fluid conducting spaces exist in the form of stromal sheets between nests of tumor cells. In this stromal network, blood vessels are embedded. We postulate that this extracellular matrix tissue represents a "fluid conducting meshwork".

INTRODUCTION

Malignant tumor growth, survival and metastasis is facilitated by several different factors including angiogenesis.[1] The role of the vasculature in mediating metastatic spread has been supported by studies demonstrating that microvessel density is a negative prognostic factor in several types of tumors,[2,3] although in other types of tumors this could not be confirmed.[4-8] Concerning both uveal and cutaneous melanoma, the relation between microvascular density and clinical outcome is controversial.[9-17] In uveal melanoma, also certain patterns of extracellular matrix deposition (i.e. arcs and back-to-back loop networks) were related to rate of metastasis and, hence, prognosis.[9,10] Initially, these patterns, identified by conventional periodic acid-Schiff (PAS) staining, were believed to represent blood vessels. Foss et al.[18] re-evaluated this issue and concluded that, instead, they consisted of connective tissue in which blood vessels were present at certain locations only, the so-called fibrovascular tissue. However, it was questioned whether the patterns described by Foss et al. were identical to the patterns studied by Folberg et al.[19]

Recently, Maniotis et al.[20] suggested that melanoma cells themselves could form a new type of vessel: blood-conducting channels lined by tumor cells, that were present in the PAS-positive back-to-back loop networks in both aggressive uveal and cutaneous melanoma. They could also be detected angiographically, indicating that channels and the normal blood vasculature were interconnected. This newly described phenomenon was termed 'vasculogenic mimicry'. A recent report [21] demonstrated the presence of "mosaic" blood vessels in which endothelial cells and tumor cells alternatingly form the luminal surface.

These data have great implications: the existence of blood conducting channels lined by tumor cells or blood vessel walls consisting out of mosaics of tumor and endothelial cells challenges the current concept stating that a tumor is fully dependent on angiogenesis for growth and metastasis.[2] In addition, tumors with vascular channels or mosaic blood vessels would be less sensitive to anti-angiogenic or anti-endothelial drugs.

Thus, elucidation of the nature of the extracellular matrix patterns is appropriate. Therefore, we set out to evaluate this issue in primary uveal melanoma. Since previous studies [22,23] showed that arc-like patterns of matrix deposition are also present in xenografts derived from human cutaneous melanoma cell lines, we re-examined this tissue as well.

MATERIALS AND METHODS

Primary uveal melanoma

Formaldehyde-fixed paraffin-embedded tissues from 30 human primary uveal melanoma cases were obtained from our archive. Uveal melanomas varied from 0.5 to 2.5 cm in diameter (median 1.7 cm) and included 28 choroidal and 2 ciliary melanoma lesions. Using H&E staining on paraffin sections, uveal melanomas were classified as 10 spindle cell type, 5 epithelioid and 15 mixed type. No information on clinical outcome was available.

Xenografts in nude mice

Human melanoma cell lines 1F6 (non-aggressive), Mel 57 (aggressive) [24] and Mel57, stably transfected with VEGF (Mel57-VEGF) (constructed by dr. W.P. Leenders, our laboratory) were cultured as previously described.[25] For induction of tumor growth, 2.5 x 10^6 cells were injected s.c. into BALB/c nu/nu mice. A number of 5 (1F6, Mel57-VEGF) or 10 (Mel57) animals were included. Subcutaneous xenografts developed in 17 mice. In the remaining 3 mice (1 injected with Mel57 and 2 mice with Mel57-VEGF cells) extensive intraperitoneal outgrowth occurred. Subcutaneous tumor volumes were estimated by multiplying length, width and height. When the tumors reached sizes between 100 and 700 mm^3, mice were injected i.v. with 100 µl of a 3% (w/v) solution of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) (12 mol FITC/mol BSA, Sigma, Brunschwig, Amsterdam, The Netherlands). Tumors were excised 60 minutes after injection and divided in three equal parts: one part was formalin-fixed, the second part was divided in two fragments that were fixed by either glutaraldehyde or periodate-lysine-2% paraformaldehyde. The last part was snap-frozen in liquid nitrogen.

(Immuno)histochemistry

Markers are listed in Table I: CD31 (JC/70A), CD34 (QBEnd/10), H and Y antigens (BNH9), thrombomodulin (anti-thrombomodulin) (all from DAKO, Glostrup, Denmark), CD31 (Mec 7.46) (Hycult Biotechnology, Uden, The Netherlands), 9F1 (Dr. A. Hamann, Hamburg, Germany) and ASD-13 (Dr. K.J.M. Assmann, our laboratory). UEA-1 (DAKO, Glostrup, Denmark), polyclonal antibodies to FVIIIrA (vWF) (CLB, Amsterdam, The Netherlands) and laminin (E2 EHS) (Dr. J. van den Born, Department of Nephrology, UMC Nijmegen) were used. Macrophages were detected by FA/11 mAb 26 (Dr. M.J. Smith, Neurobiology Division, MRC Laboratory of Molecular Biology, Cambridge, England).

Table I. Endothelial markers used for immunohistochemistry

Antibody	Antigen	Type of antibody	Tissue examined
JC70A [45,46]	CD31	mAb*	uveal melanoma
QBEnd/10 [46,47]	CD34	mAb	uveal melanoma
anti-vWF [18]	vWF (FVIIIrA)	PAbs†	uveal melanoma xenografts
BNH9 [46]	H and Y antigens	mAb	uveal melanoma
anti-Thrombomodulin [48]	Thrombomodulin	mAb	uveal melanoma
UEA-1 ‡ [18]	-	PAbs	uveal melanoma
MEC 7.46 [49]	CD31	mAb	xenografts
9F1 [24]	unknown	mAb	xenografts
ASD-13 [50]	unknown	mAb	xenografts

* mAb: monoclonal antibody
† PAbs: polyclonal antibodies
‡ lectin stained by using peroxidase-linked rabbit anti-UEA-1 PAbs

Serial paraffin-embedded 4 µm sections were stained by H&E, PAS and Azan histochemistry and by immunohistochemistry using polyclonal antibodies to laminin and markers shown in Table I. The distribution of endothelial (Mec 7.46), matrix (laminin) and macrophage (FA/11) markers was evaluated using

a standard three-step ABC method and development in 3-amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). Sections were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature and mounted in Imsol-mount medium (Klinipath B.V., Duiven, The Netherlands).

(Immuno) fluorescence and confocal microscopy

Serial 4 µm cryosections of xenograft tissue were fixed in acetone for 10 minutes. Subsequently, binding of Mec 7.46 mAb or anti-laminin antibodies was detected by secondary tetramethylrhodamine isothiocyanate (TRITC)-labelled antibodies (Alexa Fluor 568, Molecular Probes, Leiden, The Netherlands) and mounted in Vecta Shield (Vector Laboratories, Inc. Burlingame, USA). FITC-labelled BSA was visualised in these sections as well. For confocal microscopy 20 µm cryosections of Mel 57 and Mel 57-VEGF xenografts (n=3) were fixed in acetone for 10 minutes and stained by a secondary TRITC-labelled anti-rat antibody to detect Mec 7.46 binding and a secondary Cy 5-labelled anti-rabbit antibody (Amersham, Pharmacia Biotech UK Limited, Buckinghamshire, U.K.) to detect laminin.

(Immuno)electron microscopy

The endothelial marker 9F1 (Table I) and laminin were visualised in Mel 57 and Mel 57-VEGF xenografts (n=2) both by light and immuno-electron microscopy as described previously.[27,28] 9F1 mbinding was detected using DAB and anti-laminin polyclonal antibody binding using ultra small gold particles.

RESULTS

Evaluation of extracellular matrix patterns in uveal melanoma and xenografts

Vessel detection was compared in sections of uveal melanoma lesions. The anti-CD34 mAb consistently stained the vasculature (identified by morphological characteristics) in all areas of the tumor and was superior to endothelial detection by other antibodies (data not shown). For paraffin-embedded xenograft tissue, endothelial staining by ASD-13 was superior to anti-vWF, 9F1 and Mec 7.46 antibodies (not shown). Mec 7.46, 9F1 and ASD-13 mAbs immunostaining of frozen sections of various normal mouse tissues and xenografted tumors demonstrated the specificity of these mAbs for endothelial cells (not shown). Further evaluation of the vasculature on paraffin sections was therefore performed using the anti-CD34 mAb for uveal melanoma and ASD-13 mAb for xenografts. None of the used antibodies stained melanoma cells. In addition, Mel57 cells grown in vitro on microscopic slides did not express endothelial markers (not shown).

Azan staining highlighted extracellular matrix in all 30 uveal melanomas and 20 xenografts corresponding to the earlier described PAS-positive patterns,[9,10,18] which was confirmed by PAS staining of serial sections (figure 1). Erythrocytes intensely stained red (figure 2a,c (appendix)).

In 14 out of 30 uveal melanomas (47%) arcs, loops and network patterns were present confirming earlier results.[9,10] In all 30 melanomas, erythrocytes were localized in circumscript lumina in the extracellular matrix which were invariably identified as blood vessels by endothelial CD34 staining and

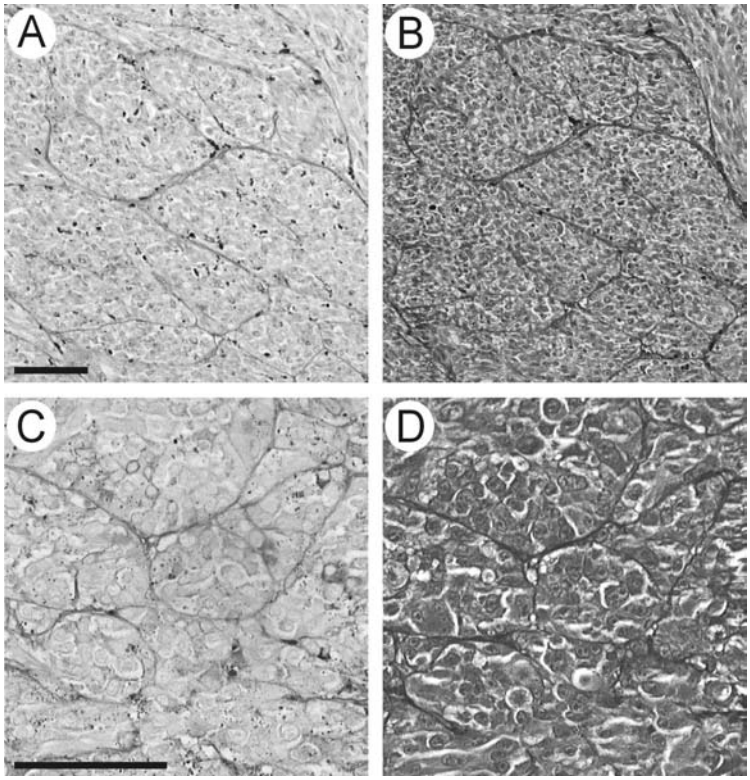


Figure 1. Evaluation of extracellular matrix patterns by PAS (**a**,**c**) and Azan histochemistry (**b**,**d**) in a human primary uveal melanoma (**a**,**b**) and xenografts of cutaneous melanoma (**c**,**d**) in serial paraffin-embedded sections. Bar = 0.1 mm.

morphological characteristics in parallel sections (figure 2a,b). Certain parts of the arced patterns were also detected by the anti-CD34 mAb, but these were not associated with closed loops, lumina or erythrocytes. Occasionally, erythrocytes were observed outside vessels in areas with hemorrhage and/or necrosis. Otherwise, neither evident circumscript lumina lacking endothelial CD34 staining nor erythrocytes outside the blood vasculature could be localized.

All xenografts (including liver and peritoneal metastasis) of human melanoma cell lines Mel57 and Mel57-VEGF contained similar arcs, loops and network patterns [10,18] as observed in the 14 uveal melanomas (see above) (figure 1). Occasionally, loops surrounded smaller groups of tumor cells and were more abundantly present than in uveal melanoma.

Xenografts of the less malignant 1F6 cell line contained an organised structure of extracellular matrix arranged in parallel patterns between groups of tumor cells.[10,18] As in uveal melanoma, erythrocytes were exclusively present in lumina surrounded by ASD-13 positive blood vessel endothelium (figure 2c,d). Especially in the Mel57-VEGF xenografts, necrosis and hemorrhage were observed in the tumor center. Note that necrosis in human uveal melanoma was hardly observed. Other evident morphological differences with Mel57 wild type xenografts were not observed.

Tumor perfusion in xenografts

To study tumor perfusion, we injected FITC-BSA as a tracer i.v. into nude mice carrying melanoma xenografts. The distribution of endothelium and laminin was identical to what was observed by endothelial immunostaining and PAS and Azan histochemistry (figure 1, 2a-f and 3c,d). Dots of laminin were also observed inside tumor cell nests. In addition, laminin was also present in PAS and Azan-positive patterns in the primary uveal melanoma (figure 3a,b). In more detail, the amount of laminin related positively with the diameter of the depositions. Based on these findings, we were able to compare the distribution of tracer in relation to the extracellular matrix patterns using fluorescence and The tracer was located exclusively within the vascular lumina in the 1F6

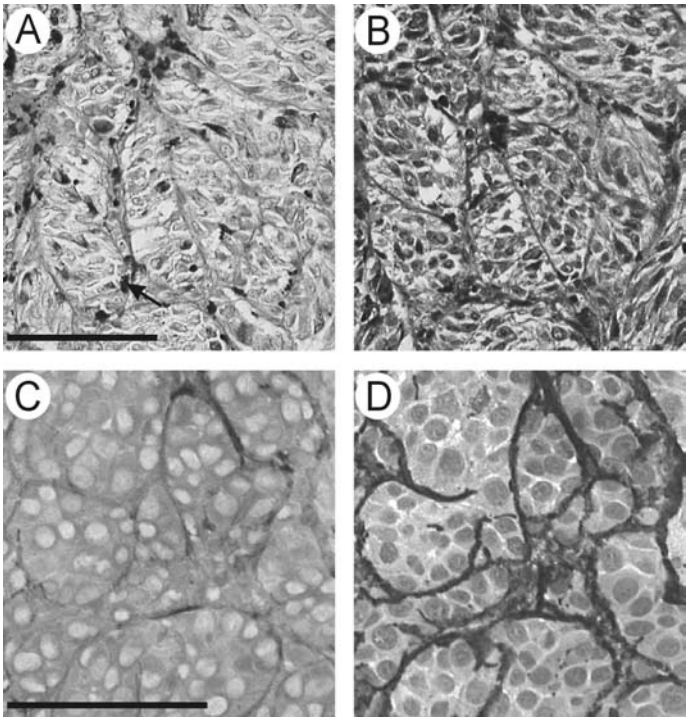


Figure 3. Evaluation of extracellular matrix patterns by PAS (**a**), laminin (**b**,**d**) and Azan (**c**) (immuno) histochemistry in human primary uveal melanoma (**a**,**b**) and xenografts of cutaneous melanoma (**c**,**d**) in serial sections. Melanin-laden macrophages (melanophages) (white arrow) are located along the PAS and laminin positive extracellular matrix patterns. Bar = 0.1 mm.

xenografts (not shown). However, in Mel57 and Mel57-VEGF xenografts, tracer was present both in and outside blood vessels in different tumor areas. Considerable amounts of tracer were found in the extracellular matrix present between tumor cell nests (arcs, loops and network patterns [10,18]) as shown by laminin staining (figure 2e-i). In xenografts of Mel57-VEGF, fluorescence intensity was slightly increased compared to xenografts of Mel57, and tracer also penetrated a larger area around the blood vasculature. In the extracellular matrix, laminin deposition enclosed tumor cell nodules (figure 2f). Often, some space was present between layers of laminin bordering tumor cells (figure 2h-i). Additional evaluation of 20 µm cryosections by confocal microscopy also failed to produce evidence for a network of open channels in these laminin-containing structures. Instead, regular patterns of matrix deposition were observed, similar as shown in figure 1f. So, these results indicated that laminin was distributed as septa surrounding nodules of tumor cells and that, where two laminin-enveloped tumor nests lie adjacent, extra space between the laminin layers may be present.

(Immuno)electron microscopic examination of extracellular matrix patterns in xenografts

Despite the presence of tracer in the extracellular matrix patterns outside blood vessels, we did not observe lumina containing erythrocytes and lacking endothelial staining at such sites. The nature of the spaces between the sheets of laminin was unclear. Therefore, 4 xenograft lesions (2 Mel57, 2 Mel57-VEGF) were processed for electron microscopy and evaluated. In agreement with our light and immunofluorescence microscopy findings, connective tissue containing large amounts of collagen, often surrounded by laminin depositions, was located between (groups of) tumor cells (figure 4b-e) in which at certain locations, lumina containing erythrocytes were present (figure 4f-g). Although it is known that tumor cells, pericytes or stromal cells cannot always be clearly differentiated from endothelial cells lining lumina in tumors by (immuno) electron microscopy,[29] additional immuno-electron microscopy using 9F1 mAb (figure 4f) indicated that lumina containing erythrocytes were lined by endothelial cells. These lumen lining cells were morphologically different from

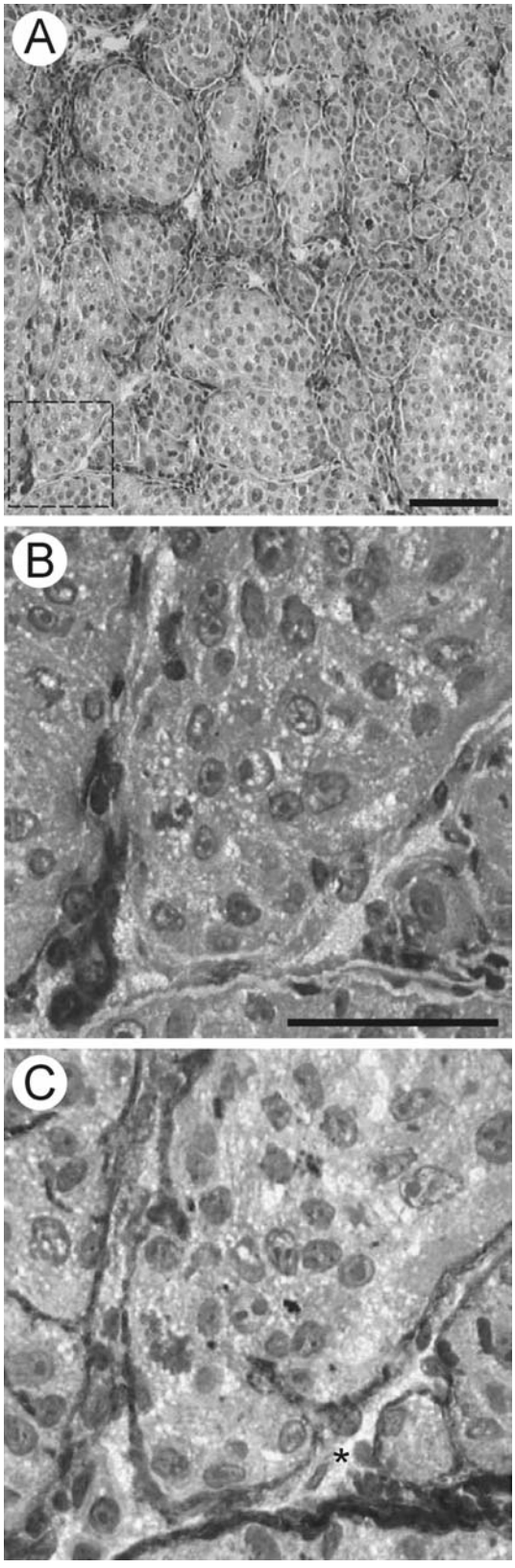


Figure 5. Immunohistochemical evaluation of the presence of macrophages in cryosections of a Mel57-VEGF xenograft. Macrophages, detected by FA/11 immunostaining, were abundantly present and closely associated with the extracellular matrix sheets (**a**, bar = 0.1 mm). Magnification of the boxed area (**a**) shows that macrophages (**b**) were located between the laminin positive sheets surrounding tumor cell nests (**c**) (**b**,**c**, bar = 50 µm). A blood vessel lumen is indicated by the asterisk (**c**) as could be detected by anti-mouse CD31 immunohistochemistry (not shown). All sections were counterstained with Harris' haematoxylin.

neighbouring tumor cells and had endothelial characteristics such as Weibel-Palade bodies and tight junctions (figure 4g-h). Lumina were always surrounded by rims of (likely endothelial) cytoplasm, which were separated from tumor cells by a basal membrane and often also by extracellular matrix (figure 4g). On the basis of these observations, these lumina were classified as putative pre-existent or extracellular matrix-associated new blood vessels. Only in case of hemorrhage or necrosis, erythrocytes were seen outside these lumina. No other true lumina besides the described lumina could be detected in the connective tissue present between the (nests of) tumor cells. No evident fenestrae between endothelial cells or transendothelial holes were observed.

Different cell types besides tumor cells were associated with the extracellular matrix patterns. Morphological analysis suggested the presence of macrophages (figure 4b), and endothelial cells (figure 4f). The presence of macrophages could be confirmed by additional immunostaining. Macrophages were mostly located between the laminin positive sheets surrounding tumor cell nests (figure 5) and present in areas of necrosis. In addition, melanin-laden macrophages were also associated with laminin-positive sheets in uveal melanoma (figure 3a,b) confirming earlier observations.[30]

DISCUSSION

Maniotis et al.[20] observed endothelial characteristics of tumor cells lining channels which is supported by the recent report by Bittner et al.[31] showing that mRNA transcripts of CD34 and other endothelial markers are expressed in melanoma cells. In this respect, the question arises whether the CD34-positive structures are blood vessels, mosaic vessels (i.e. lined by endothelial and tumor cells [21]), channels lined by tumor cells, extracellular matrix or a combination of these (for review see ref. 32). Although there are limitations in identifying endothelial cells in histological sections [32] and we did not double-stain melanoma cells for melanoma and endothelial markers, no evident positivity of any included endothelial marker (Table I) was observed on melanoma cells using light microscopy. Therefore, the identity of non-luminal CD34 positive cells is uncertain, but they may represent vascular channels lined by tumor cells expressing CD34.[20,31] So, by using of CD34 as a marker for endothelial cells in primary uveal melanoma, we were not able to confirm or rule out the existence of vascular mimicry [20] in primary uveal melanoma.

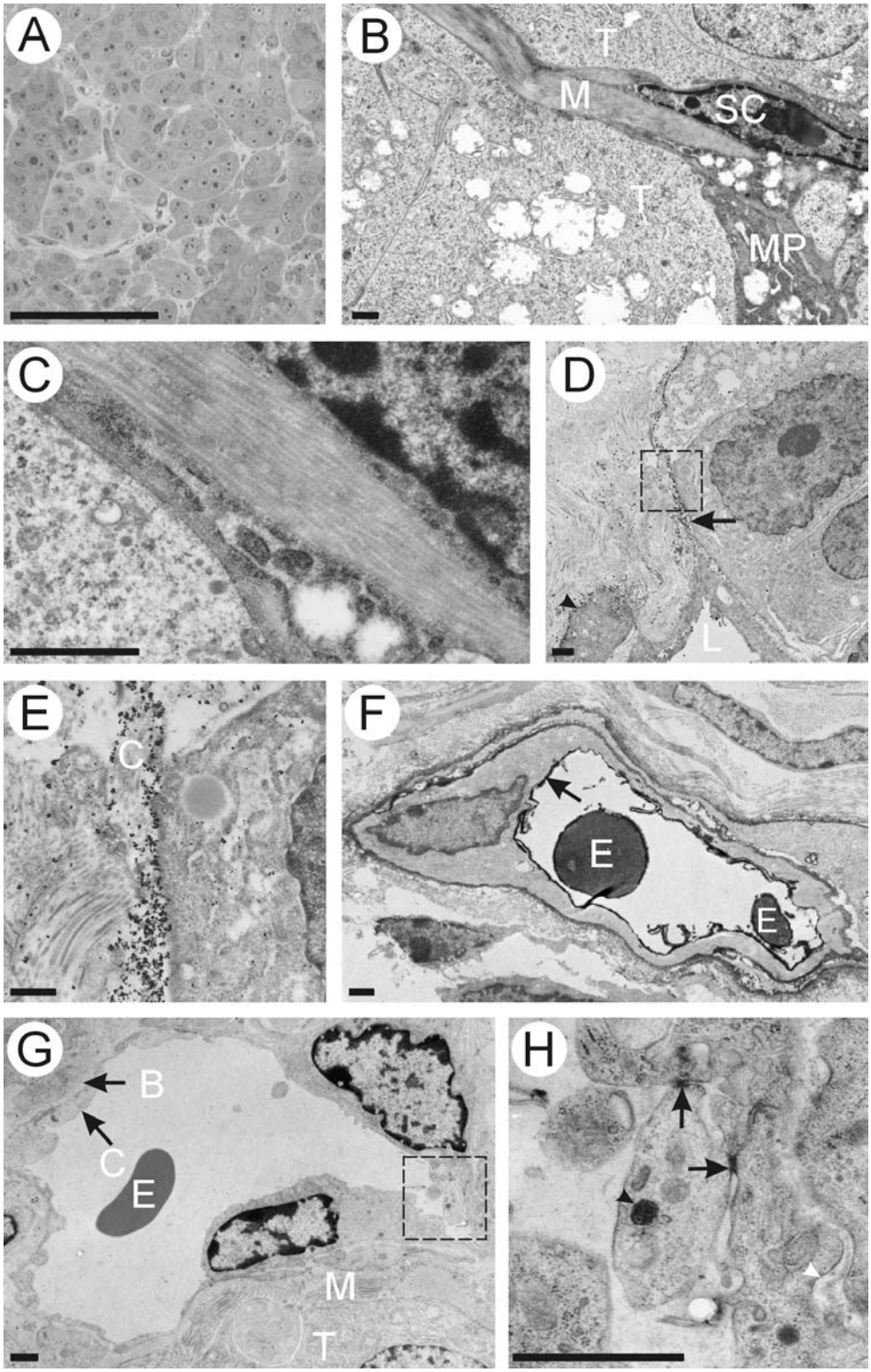
Except in the less malignant 1F6 xenografts, i.v. injected tracer could be found outside the blood vasculature in arcs, loops and network matrix [10,18] in similar patterns as observed after angiography by Maniotis et al.[20] and others.[22] These findings indicate that besides blood vessels, spaces accessible to fluids must be present in the extracellular matrix whenever these specific matrix patterns are formed. Azan staining and electron microscopy, however, failed to detect endothelium-free channels or open spaces, whereas laminin and macrophage staining indicated the existence of a compartment in which tracer and macrophages were both present. Additional evaluation of laminin deposition in sectional scanning by confocal microscopy demonstrated the presence of regular matrix patterns, deposited closely around nests of tumor cells like curved sheets. Laminin is just one of the components of the extracellular matrix. The 'spaces' visible in laminin-immunostainings contain other extracellular matrix components such as collagen (which is clearly shown in figure 4). The conduction of the i.v. administrated tracer is therefore explained by spaces in the extracellular matrix (through the network of collagen, laminin and other components) which are not visible in electron microscopy and are not observed in this study to contain blood cells such as erythrocytes. Our observations indicate that extracellular matrix is deposited as curved sheets around nests or nodules of tumor cells. In these structures, blood vessels are embedded. The extracellular matrix pattern is present between spheres of tumor cells, probably deposited as envelopes, resulting in the arcs, loops and network patterns, detected in 2D-analysis. If two spheres of tumor cells lie apposed, either one layer of matrix may be formed or two layers can be deposited separately. Between these two different septa, matrix material is localised consisting of different components (including laminin and collagen [33]) in which (limited) flow may occur (as demonstrated by the appearance of the tracer), and where different types of cells (including macrophages) are present. On the basis of these considerations, we postulate that, in fact, the extracellular matrix arcs, loops and network patterns represent a meshwork.

The presence of the tracer outside the vasculature in the extracellular matrix pattern indicates a close relationship with blood vessels. This relationship is

Figure 4. (Immuno)electron microscopy analysis of Mel57 (**b**-**f**) and Mel57-VEGF (**g**,**h**) xenograft lesions. In **a**, a low-powered overview is shown (toluidin-blue-stained 1 micron section of Mel57 xenograft tissue). A network is defined as the presence of at least 3 loops.[10] In **b**, tumor cells (T) are separated by a layer of matrix (M). The central part of **b** is shown in detail in **c**. Matrix consisting mainly of collagen fibers is present whereas no evident lumina are visible. Immuno-electron microscopy shows the deposition of laminin in basal membranes of blood vessels (**d**, arrowhead, lumen indicated by L). These layers continued into the extracellular matrix sheets (**d**, arrow), surrounding bundles of collagen (**e**, C) (indicated box in **d** is magnified in **e**). Different types of stromal cells (SC), including macrophages (MP, **b**) were associated with these layers. The nature of other stromal cells (**b**, SC) was identified by 9F1 immuno-electron microscopy (arrow) (**f**). 9F1 immunostaining demonstrated that lumina present in the extracellular matrix patterns represent blood vessels lined by endothelium, occasionally containing erythrocytes (E). Neither evident lumina, nor erythrocytes were found in the extracellular matrix besides blood vessels. Further support for the endothelial nature of the lumen-lining cells is provided in figure **g** and **h**: the lumen is lined by a rim of cytoplasm (C, **g**) which is separated from the tumor cells (T, **g**) by a basal membrane (B, **g**) and often matrix depositions (M, **g**) were present between the tumor and endothelial cells. The right part of the lumen wall (boxed area) is shown in more detail in **h**. Typical endothelial characteristics (i.e. tight junctions (arrows), Weibel-Palade bodies (arrowhead) and a basal membrane (white arrowhead) are present. Bar in **a**: 0.1 mm; in **b**-h: 1 µm.

confirmed by the enhanced leakage of tracer in the extracellular matrix in xenografts of the VEGF-transfected cell line, which is due to vascular hyperpermeability.[22] Tracer distribution may simply be explained by two hypotheses. On one hand, it is possible that spaces in the extracellular matrix generated by tumor cells [34] connect to blood vessels and are organized as channels, which was suggested previously.[20,35] In that case, non-migratory blood cells like erythrocytes would be localized in this tissue outside the vasculature, which we did not observe in the xenografted melanoma. On the other hand, interstitial spaces present in the extracellular matrix tissue (i.e. meshwork) may allow limited flow of fluid by (enhanced) leakage or permeability of the endothelium out of the blood vessels into the surrounding tissue. Considering our results, the latter hypothesis is the more likely, at least in the animal model, and the extracellular matrix arcs, loops and network patterns may thus represent a fluid-conducting meshwork.

In general, fluid movement across vessel walls is governed by differences between blood pressure and local interstitial fluid pressure (IFP). Although IFP is elevated and close to microvascular pressure in different types of solid tumors including melanoma,[36-39] our and other studies [20,22] indicate that fluid can leave the blood stream and form an exudate (without non-migratory blood cells) that moves across the microvascular arterial wall into the extracellular matrix meshwork tissue. The fluid movement is induced by the local higher arterial pressure compared to the IFP. The exudate may be conducted by the meshwork towards the venous microvasculature, where pressure is likely to be lower than the elevated IFP. This difference in pressure would allow re-entry of the interstitial fluid into veins. Alternatively, interstitial fluid may leak into surrounding pre-existent tissue (which has an IFP of approximately 0 mm Hg [37,39]) or vitreous body (oozing out) in case of uveal melanoma. High IFP is maintained by the absence of lymphatics [40] and by a limited drainage capacity of the fibrous vascular network. Comparison of the kinetics and composition of the fluid stream and composition of the extracellular matrix to that in normal



tissues could yield insight in the role of this phenomenon in tumor biology. The subcutaneous space normally is essentially avascular, however, and cannot be taken as a standard tissue. Nevertheless, our previous data indicated that tracer distribution into subcutaneous tumor tissue is relatively slow, requiring up to 45 minutes to accomplish.[22]

The extracellular matrix also harboured different types of cells, including macrophages which were located between sheets of extracellular matrix

surrounding nests of tumor cells. This observation supports the idea that migratory cells leave tumor vessels and invade by adhering to matrix components present in stromal sheets, thereby migrating into the spaces between them. This hypothetical mechanism is of significant importance since tumor-associated inflammatory cells play an evident role in tumor survival. For example, macrophages are associated both with angiogenesis and a poor prognosis in different tumor types including melanoma.[30,41-43] If macrophages are able to invade the tumor site by using the extracellular matrix, it is also possible that tumor cells can enter the blood stream via the same route. Furthermore, by formation of extracellular matrix networks, uveal melanoma could acquire an alternative system to drain excess tissue fluids, as a substitute for a lymphatic system which is absent in this type of tumor.[40] Also, the presence of the tracer in the extracellular matrix patterns indicates that nutritional components of the blood can reach tumor cells located at large distance of blood vessels. Indeed, the arcs, loops and network patterns are abundantly present in those areas in which blood vessel density is low.[20] Most data of the present study were obtained in an animal xenograft model. In this model, however, matrix patterns are formed that have evident morphological similarities with the matrix patterns present in primary uveal melanoma. Therefore, these considerations may support and explain why uveal and cutaneous melanoma containing the arcs, loops and network extracellular matrix patterns are associated with a poor prognosis.[9,10]

The close parallels such as Azan- and PAS-positivity, and the association of laminin and macrophages with these patterns highly suggest the presence of a fluid-conducting meshwork in primary uveal melanoma. In this respect, the existence of vascular mimicry [20,35] is of significant importance. Although we did not observe convincing evidence of the presence of vascular channels in the xenografted melanoma on one hand and we can not rule out their presence in uveal melanoma on the other hand, it is well possible that these blood-conducting channels and a fluid-conducting meshwork are both present in primary uveal melanoma. Furthermore, since the raised doubt about the nature of the PAS-positive patterns [9,10,18,19] and the existence of vascular mimicry,[20,35,44] the present study may further point at the true identity of the patterns. Additional study, however, is required to elucidate the exact nature of extracellular matrix patterns in uveal melanoma.

In conclusion, we propose that both in aggressive uveal and cutaneous melanoma, extravascular spaces are present besides the normal blood vasculature. These spaces are located between bordering curved septa of extracellular matrix, surrounding nests or spheres of tumor cells representing a fluid-conducting meshwork and contain different types of cells, including macrophages. There are strong indications that the arcs and loops forming the extracellular matrix are involved in inflammatory cell invasion, nutrition of tumor cells, regulation of local tissue fluid flow and, possibly, metastasis. Indeed, a fluid-conducting meshwork may represent an alternative to a lymphatic system in uveal melanoma.

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6 Functional and morphological analysis of the fluid-conducting meshwork in xenografted and primary uveal melanoma

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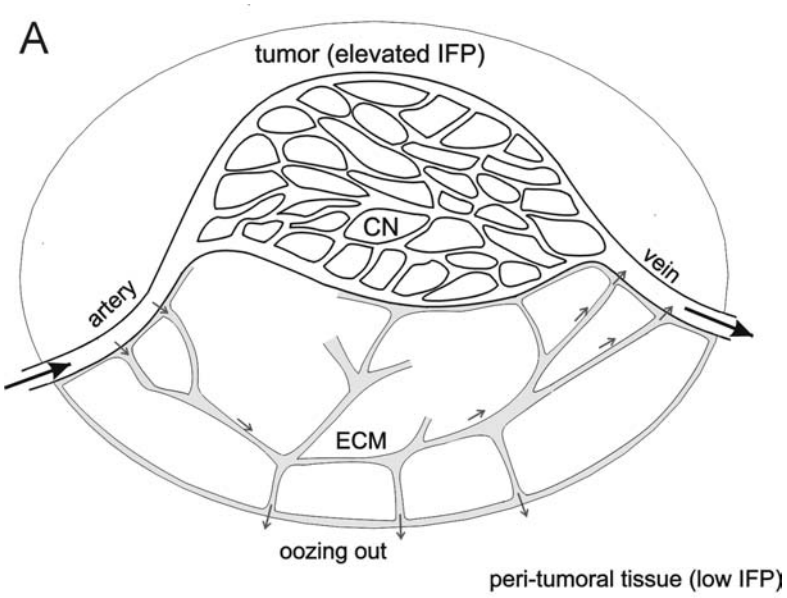
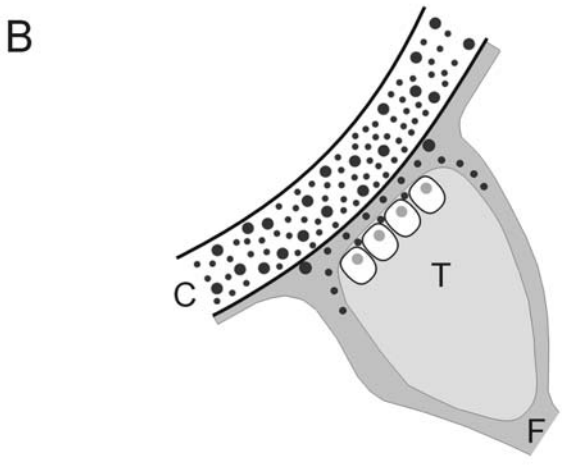
ABSTRACT

In uveal melanoma, frequently a network of extracellular matrix and capillaries forms as 'fibrovascular septa'. Follow-up data indicate that these septa negatively influence prognosis, but the pathomechanism behind this effect is unknown. We recently proposed that these structures represent a fluid-conducting meshwork which is different from the earlier described vascular channels. Evaluation of the septa in human melanoma xenografts showed that intravenously injected tracer molecules crossed the vessel wall and spread along the extracellular matrix patterns dependent on size. Murine host cells were found restricted to the matrix compartment. Our data suggest that this matrix meshwork accommodates transport of plasma-derived molecules, e.g. nutrients, to the tumor, thus enhancing growth and progression, and facilitates infiltration by host-derived cells.





INTRODUCTION

In both primary uveal and cutaneous melanoma, nine different patterns of extracellular matrix (ECM) deposition have been identified by conventional periodic acid-Schiff (PAS) staining.[1-4] Analysis of prognostic relevance indicated that the PAS-positive arcs, loops and network patterns are associated with tumor progression and subsequent poor survival.[1-5] A few years ago, it was suggested that these patterns contained or represented blood-conducting channels lined by tumor cells instead of endothelial cells (vasculogenic mimicry).[6] Since then, nature of the PAS-positive patterns and the existence of vasculogenic mimicry have become subjects of extensive debate.[6-10]

Recent studies reported the presence of laminin, collagen I and VI in uveal melanoma,[9,11] and different types of cells including endothelial cells and macrophages along the PAS-positive arcs, loops and network patterns.[9,12,13] Macrophages in malignant tumors, including uveal melanoma, have been associated with adverse prognosis,[12-16] although their function is unknown. Heparan sulfate proteoglycans (HSPGs) and other structural proteins which are key components of the ECM, may also be of significant importance for tumor growth and metastasis, but their exact role is unclear as well.

In a recent study,[9] we re-assessed the nature of the PAS-positive patterns in primary uveal and xenografted melanoma. We were not able to confirm the presence of blood-conducting channels, but our findings are in line with data of Foss et al.[17] showing that the patterns constitute fibrovascular septa. Furthermore, we suggested that a fluid stream might be present in these ECM depositions apart from the blood vasculature. This "fluid-conducting meshwork" (figure 1a) was found in areas with a relatively low vascular density but without evident necrosis,[7] indicating that it might play a role in tumor cell nutrition. Obtaining knowledge of the functional properties of this presumed fluid-stream is essential to understand the mechanisms of tumor outgrowth and metastasis, and may be helpful in designing more efficient diagnostic and therapeutic intervention.

To elucidate the properties of the ECM arcs, loops and network patterns, we have now analyzed their ability to transport tracer molecules, details of their composition and the presence of associated cell types in xenografted cutaneous and primary uveal melanoma.

Figure 1. Schematic drawing of a hypothetical mechanism of fibrovascular tissue perfusion in tumors. In a, blood flow is conducted by the artery, via the capillary network (CN) towards the venous vessel (large arrows). Exudate-like fluid moves across an arterial microvessel wall (small arrows) into the extracellular matrix (ECM) in tumor areas where arterial pressure exceeds interstitial fluid pressure. ECM patterns conduct the exudate-like fluid towards the venous microvasculature (small arrows) where blood pressure is lower than the elevated interstitial fluid pressure,[26] causing re-entry into the blood stream. Also, leakage into peri-tumoral tissue (in which the interstitial fluid pressure is approximately 0 mm Hg 39) or the vitreous body in case of uveal melanoma (i.e. oozing out) may occur. The boxed area (**a**) is magnified in **b**, showing leakage of the exudate-like fluid containing small and large sized molecules from the blood vasculature into the ECM compartment. Deep infiltration of large sized molecules is limited. Only small sized molecules are able to penetrate fully into the ECM patterns and also in small amounts between the tumor cells lining these patterns (T, tumor; C, capillary; F, fibrovascular septa).

MATERIALS AND METHODS

Uveal melanoma

Frozen specimens of 6 uveal melanoma lesions were obtained from the pathology archives of the University Medical Center Nijmegen where they were stored at -130^0C. Tissues were obtained according to guidelines of Dutch legislation. All specimens had been diagnosed by a pathologist and were stained by Azan histochemistry for visualization of the extracellular matrix patterns.[9,18] Three melanomas contained the arcs, loops and network patterns and were selected for further immunohistochemical study.

Xenografts in nude mice

The human cutaneous melanoma cell line Mel57 [19] was cultured as previously described.20 For the induction of tumor growth, 2.5 x 10^6 cells were injected s.c. into BALB/c nu/nu mice (n=83). Subcutaneous xenografts developed in all mice as a result of the tumor cell injection. Subcutaneous tumor volumes were estimated by multiplying length, width and height. When the tumors reached sizes between 100 and 700 mm^3, mice (n=80) were injected i.v. with 100 µl of tracer solution (see below). Tumors were excised at 2, 10, 15, 30, 45 minutes or 1, 3 or 24 hours after injection and snap-frozen in liquid nitrogen. In xenografts, tumors were divided in two parts: one part was snap-frozen in liquid nitrogen and the other part was formalin-fixed. Two animals were included per tracer type and time point.

Tracers

Tracers with a broad range of molecular size were chosen. A 3% (w/v) solution of bovine serum albumin (BSA) labeled with fluorescein isothiocyanate (FITC-BSA, molecular weight approximately 68 kDa) (12 mol FITC/mol BSA), with sulfo-rhodamine 101 acid chloride (Texas Red-BSA) (3 mol Texas Red/mol BSA) or with biotin (8-16 mol biotin/mol BSA) was injected i.v. In addition, 6% (w/v) of FITC-labeled bovine insulin (FITC-I) (1 mol FITC/mol insulin, molecular weight approximately 5.8 kDa) and 1%, 2%, 3% and 6% (w/v) solutions of FITC-labeled dextrans (FITC-D) (0.003-0.02 mol FITC /mol glucose) with average molecular weights of 20,000 (FITC-D20), 40,000 (FITC-D40), 70,000 (FITC-D70) and 2,000,000 (FITC-D2000) were also tested. We selected these tracers since they were representative for small nutrient molecules (i.e. FITC-I, FITC-D20 and 40), indicative for endothelial permeability changes (i.e. FITC-D70, labeled BSA), or assumed to be purely intravascular molecules (i.e. FITC2.000). All tracers were obtained from Sigma, Brunschwig, Amsterdam, The Netherlands.

To determine the optimal conditions for studying tumor perfusion, we first injected nude mice carrying melanoma xenografts i.v. with 3% labeled BSA, 6% FITC-I or 1, 2 ,3, 6% FITC-D70 and evaluated tracer distribution in the tumor tissue after 2 (in case of FITC-I), 30 and 60 minutes (in case of labeled BSA and FITC-D70). The distribution of FITC-BSA and FITC-I could readily be detected in all xenografts as shown previously for FITC-BSA (figure 2b (appendix)).[9,21] After perfusion with Texas Red-BSA, fluorescence was hardly detectable (not shown). Histochemical visualization of biotin-BSA was unsuccessful as well because of high background (not shown). With 6% FITC-D70 there was reproducible visualization of tracer distribution on fresh

cryosections (not shown). Therefore, 3% FITC-BSA, 6% FITC-I and 6% FITC-D solutions were selected for further study.

Table I. Primary antibodies used for immunohistochemistry.*

primary antibody	marker specificity	source of antibody
Mec 7.46	CD31	Hycult Biotechnology, Uden, The Netherlands
anti-laminin **	laminin	Dr. J. van den Born, Department of Nephrology, UMC Nijmegen, The Netherlands
3G10	pan-HSPG	[29]
JM403	heparan sulfate side chains	[30]
10H4	syndecan-2	[31,32]
2E9	syndecan-1,-3	[32,33]
1C7	syndecan-3	[32,33]
S1	glypican-1	[34,35]
JM72	agrin	[36]
1948	perlecan	Chemicon International Inc, Temecula, CA, USA
anti-all hu XVIII (QH48.18) **	both collagen XVIII variants [37]	[38]
ASO2	fibroblast	Dianova, Hamburg, Germany
L26	CD20	DAKO, Glostrup, Denmark
anti-leu-4	CD3	Becton Dickinson, San Jose, CA, USA
anti-collagen I **	collagen I	Monosan, Uden, The Netherlands
anti-collagen III **	collagen III	Monosan
anti-collagen IV	collagen IV	Sigma, Brunschwig, Amsterdam, The Netherlands
anti-collagen V**	collagen V	Monosan
HHF35	α-smooth muscle actin (pericytes)	Dako

* all antibodies were applied in uveal melanoma except for Mec 7.46 which was applied in xenografted melanoma.
** polyclonal antibodies

(Immuno) fluorescence

4 µm cryosections of xenografts containing labeled BSA were fixed in acetone for 10 minutes. Binding of anti-CD31 monoclonal antibody (MAb) Mec 7.46 (Table I) was detected by secondary tetramethylrhodamine isothiocyanate (TRITC)-labeled antibodies (Alexa Fluor 568, Molecular Probes, Leiden, The Netherlands). In the same section, binding of anti-laminin polyclonal antibodies (Table I) was detected by a secondary Cy 5-labelled anti-rabbit antibody (Amersham, Pharmacia Biotech UK Limited, Buckinghamshire, U.K.) Nuclear counterstaining was performed by incubating the sections for 1 minute in 4',6-diamidino-2-phenylindole solution (Dapi, 0,2 mg/ml) (Sigma) and mounted in Vecta Shield (Vector Laboratories Inc., Burlingame, USA). In case of xenograft perfusion with FITC-I and FITC-D, tracer distribution was directly visualized in an unfixed 4 µm cryosection. Images were digitally stored. Subsequently, sections were fixed and double-stained. Distribution of CD31 and laminin were also visualized and digitally stored. To evaluate tracer distribution, digital images before and after double staining were matched.

In situ hybridization

Three xenografted tumors were used for DNA in situ hybridization analysis. 4 µm paraffin-embedded sections were deparaffinized, rehydrated, and immersed in 3% H2O2 in phosphate-buffered saline for 30 minutes. Subsequently, sections were incubated for 10 minutes in 1M sodium thiocyanate at 80°C and protein digestion was performed for 15 minutes in pepsin dissolved in distillated water (400 U/ml, pH 2.0). Sections were dehydrated and air-dried before hybridization. Total DNA was isolated from human whole blood and murine kidney and liver tissue by using the Puregene DNA isolation Kit (Gentra, Minneapolis, USA). Human DNA was labeled with biotine-16-dUTP using a nick translation mix (Roche Diagnostics GmbH, Mannheim, Germany) and murine DNA with digoxigenin-11-dUTP (Roche Diagnostics GmbH). Both probes (each probe: 3.3 ng/µl) were mixed in hybridization buffer (62.5% formamid, 10% dextran sulphate, 2x SSC-tween, pH 7.0) and denaturated at 72.5°C for 5 minutes. Sections were hybridized overnight at 42°C. Subsequently, sections were washed at 45°C for 15 min with a solution of 50% formamid and 2x SSC (pH 7.0) and in PN buffer (10 mM/L Na2HPO4, 10 mM Na2H2PO4, 0.5% Nonidet P-40 detergent) for 10 minutes at room temperature. Biotin-labeled human DNA was detected with mouse anti-biotin polyclonal antibodies (PAbs) (diluted 1:100) (Dako, Glostrup, Denmark), alkaline phosphatase-conjungated biotin-avidin complex (Vectorstain, Vector Laboraties), and visualized by incubation with a mixture of 1 mg/ml Fast Blue, 0.2 mg/ml naphthol phosphate and 0.24 mg/ml levamisole (Sigma) for 10 minutes. Digoxigenin-labeled mouse DNA was detected using mouse anti-digoxigenin PAbs (dilution 1:50) (Roche Diagnostics GmbH), EnVision system HRP (Dako), and developed by a 10-minute incubation with a 0.4 mg/ml amino-9-ethyl-carbazole solution. Sections were mounted using Imsol mounting medium.

Immunohistochemistry

Antibodies used for immunohistochemistry are listed in Table I. The distribution of all included markers and biotin-BSA was evaluated using a standard three-step ABC method (Vectastain, Vector Laboratories) and development in 3-amino-9-ethyl-carbazole (Aldrich, Steinheim, Germany). Strongly pigmented sections were selectively bleached when necessary. For staining with the 3G10 MAb antibody, directed against heparitinase-digested HSPGs, sections were pretreated with 50 mU heparitinase (heparinase III, EC 4.2.2.8; Sigma). All sections were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature and mounted in Imsol-mount medium (Klinipath B.V, Duiven, The Netherlands).

RESULTS

Tracer studies

Tracer localization was determined by comparing its distribution with laminin (ECM patterns) and CD31 (microvasculature) immunofluorescence as described previously.[9] We found that tracer ability to enter the ECM was dependant on molecular size: FITC-BSA spread along matrix patterns outside the blood vasculature in approximately 45 minutes (not shown). FITC-I entered the patterns within 2 minutes (figure 2a-c, appendix). FITC-D70 infiltrated the patterns after a time interval similar to that observed with FITC-BSA (figure

2j), whereas FITC-D20 and FITC-D40 appeared already after 10 minutes (figure 2d-f, j). In particular with FITC-I and FITC-D20, a diffuse staining pattern was seen, indicating that this tracer was able to penetrate into the tumor nests as well. The large molecular size marker FITC-D2,000 extravasated perivascularly and was observed occasionally close to large vessels (figure 2g-h). Remarkably, at 24 hours after injection FITC-BSA was still present in the patterns whereas FITC-D of any size had completely disappeared. When we incubated a 3% FITC-BSA solution for one hour on a 4 µm unfixed cryosection of three xenografts, it appeared that FITC-BSA was able to bind to the ECM patterns directly and, to a lesser extent, to the tumor cells as well (not shown).

In situ hybridization of xenografted tumors

In situ hybridization analysis of both human total DNA and mouse total DNA showed the localization of human tumor cells and associated murine stromal cells in melanoma xenografts. Apart from intravascular white blood cells and endothelial cells, host cells were present predominantly along the ECM patterns whereas infiltration between tumor cells was rare (figure 3 (appendix)).

Table II. Immunohistochemical analysis of uveal melanoma*

extracellular matrix component	arcs, loops and network patterns	micro vasculature	tumor cells
pan-HSPG	+	+	+
heparan sulfate side chains	+	+	-
syndecan-2	-	-	-/±
syndecan-1,-3	-	-	+
syndecan-3	-	+	-
glypican-1	-/±	-	±
agrin	+	+	-
perlecan	+	+	-
collagen XVIII	+	+	-
collagen I	+	+	-
collagen III	-	±	-
collagen IV	+	+	-
collagen V	+	+	-

* Intensity of staining was scored as negative (-, figure 4a), weak (±, figure 4i) or strong (+, figure 4b).

Morphological analysis of the extracellular matrix patterns in uveal melanoma

To investigate the composition of the ECM patterns and to study effects of the presence of such patterns on the localization of infiltrating immune cells in uveal melanoma, immunohistochemical analysis was performed using a panel of well-characterized antibodies. The results are listed in Table II. Except collagen III, all tested subtypes of collagen were present in the ECM patterns in primary uveal melanoma (figure 4a-d). Basement membrane-associated HSPGs agrin (figure 4e) and perlecan (figure 4f were found in the extracellular matrix patterns and surrounding the vasculature whereas expression of the cell membrane-associated HSPGs syndecan-1,-3 (figure 4g), syndecan-2 (figure 4h) and glypican-1 (figure 4i) was weak and mainly restricted to tumor cells.

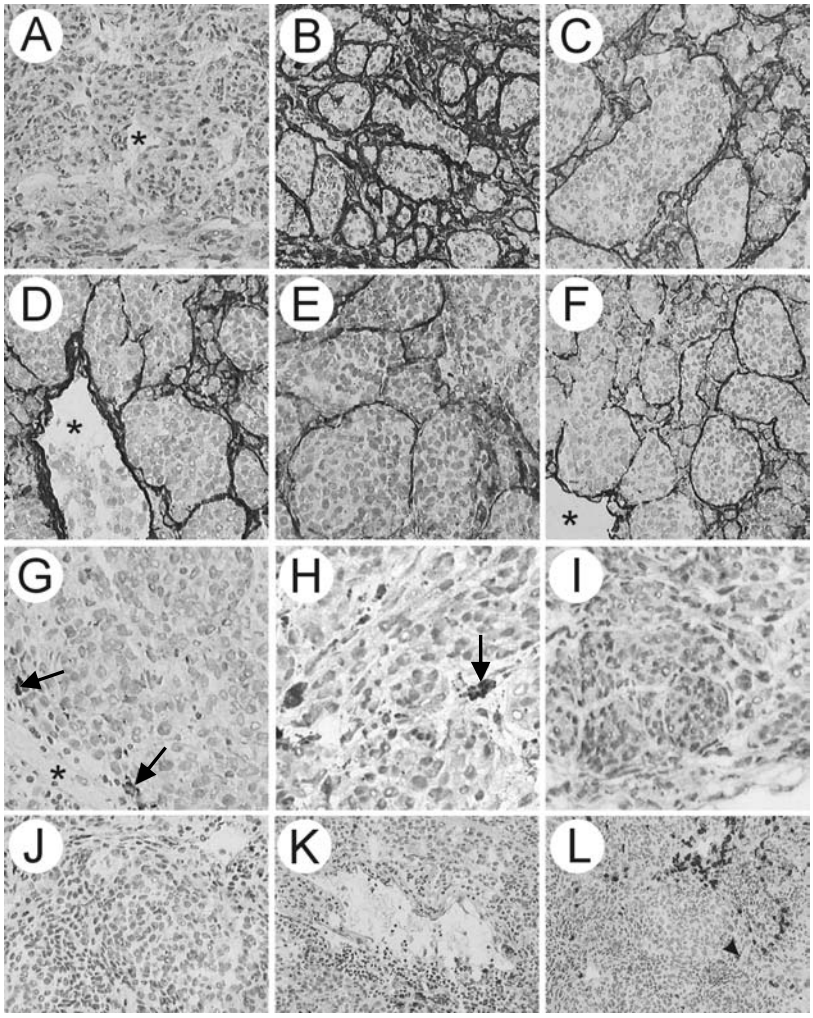


Figure 4. Immuno-istochemical analysis of the expression of collagen III (**a**), collagen IV (**b**), collagen V (**c**), collagen XVIII (**d**), agrin (**e**), perlecan (**f**), syndecan-1,-3 (**g**), syndecan-2 (**h**) and glypican-1 (**i**) and the presence of pericytes (anti-α-SM1 mAb, **j**), B-lymphocytes (anti-CD20 mAb, **k**) and T-lymphocytes (anti-CD3 mAb, **l**) in primary uveal melanoma. Blood vessel lumina are indicated by asterisks (**a**,**d**,**f**,**g**) and syndecan-positive stromal cells (possibly macrophages) by arrows (**g**,**h**). In **k**, B-cells are neighbouring a large blood vessel. In **l**, T-cells are occasionally associated with the extracellular matrix arcs, loops and network patterns (arrow). Counterstained with Harris' haematoxylin. Magnification **a**-**f**, **i**, **k**, **l**: 200x, **g**-**h**, **j**: 400x.

Occasionally, syndecan-1,-3 and syndecan-2 were expressed by pattern-associated cells, probably macrophages, as shown previously [22] (figure 4g-h). Extensive staining of the ECM patterns by a fibroblast marker (Table I) was observed. However, no individual cells could be identified in these patterns (not shown) which suggests that a truly reliable marker for fibroblasts may not be available yet. α-Smooth muscle actin-positive pericytes were mostly confined to larger vessels (figure 4j).

Infiltrate analysis showed that few B-lymphocytes were present around major vessels at sites of dense infiltration (figure 4k). T-lymphocytes were also present in the perivascular infiltrates but could occasionally be found along the patterns in uveal melanoma (figure 4l).

DISCUSSION

Theoretically, there are a number of possible explanations for the effects of the ECM arcs, loops and network patterns on tumor progression in uveal melanoma: 1) the matrix deposits facilitate angiogenesis, 2) they facilitate access of plasma-derived molecules to the tumor cells, 3) they facilitate infiltration of tumor tissue by macrophages, that, in turn, enhance angiogenesis,[23] 4) they facilitate escape of tumor cells from the primary tumor lesion and 5) matrix deposition is merely a secondary effect of tumor

progression. In line with the second explanation, we recently described that the ECM arcs, loops and network patterns present in uveal and cutaneous melanoma and in melanoma xenografts, may represent a fluid-conducting meshwork, functionally analogous to but anatomically different from the previously described vascular channels.[6,9] Functional evaluation of this meshwork by fluorochrome-labeled insulin, BSA and dextrans of different size indicated that there is a rapid entrance into the patterns of particles with a Stokes' radius of 4.4 nm of less (based on the molecular weight of FITC-D40 using the method of Granath et al.[24]) within 2-10 minutes, whereas infiltration of larger particles (Stokes' radius more than 5.8 nm (FITC-D70)) took approximately 30 to 45 minutes. In addition, some leakage of small molecular sized FITC-I and FITC-D20 out of the ECM patterns between the tumor cells lining these patterns occurred (figure 1b). The exact nature of this transport is unclear: fluid may be transported by an actual current, by diffusion and convection,[25] or through vascular channels. We did, however, not observe these channels in our animal model.[9] In addition, it is expected that the intravascular tracer FITC-D2,000 should have been outside the vasculature in larger amounts than that we observed, if vascular channels had been present. Our data are therefore in line with either a process of diffusion and convection,[25] or with a stream of molecules of limited size, comparable to pressure-driven filtration (figure 1). Entrance of fluid derived from the blood into the patterns is determined by the permeability of the endothelium and the compactness of the extracellular matrix. Leakage of FITC-D2,000 was mainly restricted to the perivascular space, indicating that entrance of particles with a Stokes' radius of at least 27.9 nm, is mainly determined by extracellular matrix composition.

Our tracer data imply that nutrients can relatively rapidly be transported via the arcs, loops and network patterns towards tumor cells located at some distance from the blood vasculature. Hence, in primary uveal melanoma the role of the fluid-conducting meshwork may be essential for survival of tumor cells and explains why necrosis is absent in areas that contain arcs, loops and network patterns but have low numbers of blood vessels.[7] Although tumor vessels are leaky to macromolecules in many experimental and human solid tumors, extravasation of these molecules is often poor. This may be explained by the high interstitial fluid pressure in the center of a tumor.[26] This phenomenon is of significant importance for the design and application of therapeutic agents. However, in case of tumors containing the arcs, loops and network patterns, these patterns may provide a gateway for the delivery of therapeutic agents into the tumor lesion. So, although the patterns may be involved in tumor cell nutrition and progression on one hand, their presence may contribute to efficient therapy using agents directed against tumor and tumor-associated cells on the other hand. In this respect, a possible effect on immunotherapy is supported by a previous study showing that intravenous injection of specific monoclonal antibodies and FITC-D in melanoma-bearing mice resulted in similar distribution patterns.[27]

In this and a previous study,[9] we have shown that the matrix-associated cells were mainly macrophages, endothelial cells and stromal cells in both uveal and xenografted melanoma. In addition, it appeared that murine cells were hardly present inside the tumor cells nests in melanoma xenografts, indicating that infiltration of hosts cells is limited to the ECM patterns. So, these data indicate

that the extracellular matrix patterns may serve as a gateway for host cells entering the tumor, and perhaps, also for tumor cells to escape from the tumor, explaining thereby the higher degree of malignancy. Furthermore, the presence of non-tumor cells along the matrix patterns raises the question whether ECM patterns are assembled by stromal cells, by melanoma cells or by both. In any case, they seem to be induced primarily by the presence of the tumor cells, and are therefore fundamentally different from a pre-existent stroma in which a tumor lesion invades. It may be hypothesized that melanoma cells deposit ECM around small nests of tumor cells, that then the deposited matrix becomes infiltrated by different types of cells, including endothelial cells, fibroblasts and macrophages, and finally, that these latter cells contribute to further remodeling, maturation and expansion of the patterns. As suggested previously,[11,28] tumors containing extracellular matrix arcs, loops and network patterns may thus be comparable to a battlefield: the neoplastic cells being the commanding officers and the stroma (fibroblasts, macrophages) the executing soldiers.

In conclusion, our data suggest that the ECM arcs, loops and network patterns accommodate the transport of plasma-derived molecules (oxygen and nutrients) into the tumor lesion, thereby enhancing tumor growth and progression and facilitating infiltration of tumor tissue by host-derived cells.

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7 EMAP-II expression is associated with macrophage accumulation in primary uveal melanoma

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SUMMARY

Primary uveal melanoma may contain arcs, loops and networks of periodic acid-Schiff (PAS)-positive patterns along which numerous macrophages are present. Their recruitment into tumor tissue is mediated by chemotactic cytokines, for which endothelial growth factor C (VEGF-C) and endothelial-monocyte activating polypeptide II (EMAP-II) are candidates. In this study, the extent of VEGF-C and EMAP-II immunoreaction was related to macrophage infiltration. Serial sections of 25 primary uveal melanoma lesions were analyzed by immunohistochemistry. Our analysis showed no correlation of VEGF-C immunoreaction and localization of macrophages. However, accumulation of macrophages occurred at sites of EMAP-II expression, especially in areas containing nests of tumor cells, surrounded with arcs, loops and network patterns. In tumors with a strong EMAP-II immunoreaction, the adhesion molecule intracellular adhesion molecule-1 (ICAM-1) was strongly expressed on endothelial cells. EMAP-II-positive endothelial cells did not express vascular endothelial growth factor receptor-2. However, extensive release of von Willebrand Factor was observed. Signs of apoptosis were found neither in tumor cells nor endothelial cells. In uveal melanoma, macrophages accumulate at sites of EMAP-II expression. Based on the results, it may be hypothesized that this process of chemotaxis is facilitated by EMAP-II-dependent expression of ICAM-1 on vascular endothelial cells and concomitantly leads to localised vascular damage as indicated by von Willebrand Factor release.

INTRODUCTION

In both primary uveal and cutaneous melanoma, nine different patterns of extracellular matrix deposition have been identified by conventional periodic acid-Schiff (PAS)-staining.[1-3] These different patterns appeared to be of prognostic significance: especially the presence of PAS-positive arcs, loops and network patterns was associated with poor survival.[1-4]

In a recent study,[5] we suggested that these fibrovascular septa constitute a fluid-conducting meshwork and contain endothelial cells, stromal cells and macrophages along the arcs, loops and network patterns. Other reports [6,7] also noted the abundant presence of macrophages in uveal melanoma. In spite of this abundance,[5-7] their role in uveal melanoma is largely unknown. Macrophages may be involved in angiogenesis (reviewed in ref. 8), which is supported by a recent study that showed that the number of macrophages was related to microvascular density.[6] High numbers of macrophages located in the tumor have also been related to poor prognosis in primary uveal melanoma [6,7] and in other tumor types.[9-11] Furthermore, their strict colocalization with the arcs, loops and network patterns [5,6] suggests either a role in the development of these patterns or, alternatively, the use of these patterns to invade the tumor lesion.

It is unknown how invasion of macrophages in uveal melanoma is mediated. As recently reported, in xenografted melanoma macrophages were recruited by vascular endothelial growth factor C (VEGF-C),[12] which is also expressed in uveal melanoma.[13] Besides VEGF-C, endothelial-monocyte activating polypeptide II (EMAP-II) may also be involved in the process of macrophage invasion because of its ability to attract monocytes and granulocytes.[14,15] EMAP-II mRNA was detected in normal and in tumor tissues.[16-21] EMAP-II was originally described as a tumor-derived cytokine, isolated from MethA tumor supernatant.[14,15] It is involved in embryonic development,[16,22,23] inflammation [21,24] and autoimmune disease.[25]

The possible effects of EMAP-II on tumor growth are complex.[26] On one hand, EMAP-II release by hypoxic or apoptotic tumor cells may be involved in macrophage influx and subsequent macrophage-mediated angiogenesis in hypoxic or apoptotic areas.[14,15,17] Indeed, hypoxic prostate adenocarcinoma cells release EMAP-II.[20] Infiltration of blood-stream monocytes in tumor tissue is mediated via endothelial adhesion proteins ICAM-1 and VCAM-1 and, to lesser extent, P- and E-selectin (for extensive review see: ref [27]). If EMAP-II expression mediates recruitment of monocytes and resident macrophages to malignant tumors, this would require concomitant expression of endothelial adhesion proteins. It was already described previously that P- and E-selectin are upregulated on endothelial cells under the influence of recombinant EMAP-II *in vitro*.[14] On the other hand, EMAP-II-mediated influx of macrophages may enhance the immune response against a tumor and thereby counteract tumor growth. Furthermore, EMAP-II may also inhibit tumor growth by targeting the tumor vascular bed via induction of endothelial cell apoptosis [18] by binding to α-ATP synthase [28] and priming the tumor vasculature for (local) destruction by TNF-α.[19,29,30] These previously proposed mechanisms were based on circumstantial evidence, however, and are therefore speculative. Most data in recent literature were obtained by studying the effects of applied, recombinant

EMAP-II. So, the effects of endogenous EMAP-II, produced in the tumor interstitium, are unclear.

In order to elucidate the role of endogenous, tumor-derived EMAP-II in primary uveal melanoma, we evaluated both the occurrence and localization of EMAP-II, ICAM-1 and VCAM-1 and determined the presence of macrophages by immunohistochemistry. In addition, levels of apoptosis, endothelial activation (as indicated by expression of KDR (also known as vascular endothelial growth factor receptor-2 (VEGFR-2 [31])) and signs of endothelial damage (as characterized by von Willebrand Factor (vWF)-release [32]) were studied. Finally, we investigated VEGF-C expression in relation to the presence and localization of macrophages.

MATERIALS AND METHODS

Patient material

Tissues were obtained according to guidelines of the Dutch legislation and our work adhered to the tenets of the Declaration of Helsinki. Frozen specimens from 25 uveal melanomas were obtained from the pathology archives of the University Medical Centre Nijmegen where they were stored at -130^0C. Presence of disease in all specimens had been determined by a pathologist. All primary melanomas were obtained by surgery and data of the clinical outcome was obtained of 16 patients with a follow-up time from 1 to 13 years (median 4.5 years). The uveal melanomas varied from 7 to 28 mm in diameter (median 22 mm) and included 23 choroidal and 2 ciliary melanoma lesions. Uveal melanoma lesions were divided in two parts along the maximal diameter. One part was formalin-fixed and the other part was snap-frozen. Using H&E staining on paraffin sections, 5 uveal melanomas were classified as 5 spindle cell type and 20 epithelioid or mixed type. Azan and PAS histochemistry without counterstaining on unbleached paraffin sections showed that 10 (44%) uveal melanomas contained loops and networks matrix patterns (figure 1a).[1,2,5,13,33] It appeared that in lesions previously classified as 'arcs, loops and network patterns',[1,2] the presence of arcs (with and without branching) is minimal. In addition, the arcs, loops and network patterns. are focally present in a tumor. Therefore, it is possible that sampling errors occurred leading to evaluation of a section containing as no network patterns whereas they were present in other parts of the tumor. Taken together, these considerations may explain the relatively low incidence of arcs, loops and network patterns in our specimen. In our previous experience,[5,13] the extent of melanin pigmentation did not significantly hamper the detection of extracellular matrix by PAS and Azan histochemistry. The use of PAS and Azan histochemistry in identifying matrix patterns was recently confirmed by immunofluorescence and electron microscopy.[5]

Antibodies

Antibodies used for immunohistochemistry are listed in Table I. Anti-CD34 (QBEnd/10), anti-CD68 (Kp1 and PG-M1) and anti-vWF were from DAKO, Glostrup, Denmark. Anti-ICAM-1 (PN-E12.1) was raised in our laboratory [34] and anti-VCAM-1 was from Immunotech S.A., Marseilles, France Anti-KDR (Clone KDR-2) was from Sigma, Saint Louis, Missouri, USA. Goat polyclonal antibodies to VEGF-C were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Rabbit polyclonal antibodies EMAP-II (SA 2846) were obtained from Dr. M. Clauss, Max Planck Institute, Bad Nauheim, Germany. The rabbit was boosted once against a purified recombinant peptide containing 20 amino acids located at the amino terminal site of the mature human EMAP-II form. Control experiments demonstrated the specificity of the antibodies to both mouse and human EMAP-II and its precursor p43 as previously shown.[17,35]

Table I. Primary antibodies used: antigens and type of cells stained

Antigen	Ab	type of cell
CD34	QBEnd/10	endothelial
EMAP-II	anti-EMAP-II *	tumor, endothelial
CD68	Kp1	macrophages
CD68	PG-M1	macrophages
VEGF-C	Anti-VEGF-C *	macrophages
ICAM-1	PN-E12.1	endothelial
VCAM	anti-VCAM-1	endothelial
vWF	anti-vWF *	endothelial
KDR	anti-KDR	endothelial

* polyclonal antibodies

Immunohistochemistry

Four micron cryosections were air-dried and fixed in acetone at room temperature for 10 minutes. Only strongly pigmented sections were bleached by incubating the sections in 3.0% (v/v) hydrogen peroxide and 1.0% (w/v) disodium hydrogen phosphate for 18 hours at room temperature. Cryosections were incubated with 3% hydrogen peroxide for 30 minutes and subsequently with 20% normal goat (EMAP-II and vWF stainings) or horse serum (other stainings) for 10 minutes. Successive sections of each specimen were incubated with QBEnd/10 (diluted: 1:100), anti-EMAP-II (diluted: 1:80) polyclonal antibodies, Kp1 (diluted: 1:100), PG-M1 (diluted 1:50), PN-E12.1 (undiluted supernatant), anti-VCAM-1 (diluted: 1:40), anti-vWF (diluted: 1:300), anti-VEGF-C (diluted 1:20) for 60 minutes at room temperature. Anti-KDR (diluted 1:400) was incubated overnight at 4^{o}C. After the first and all following incubation steps, sections were rinsed with ample phosphate-buffered saline (PBS). Then secondary, 1:200 diluted biotinylated affinity-purified anti-rabbit IgG (for the EMAP-II and vWF stainings), anti-goat (for VEGF-C staining) or anti-mouse IgG (all other stainings) (Vectastain, Vector Laboraties) was incubated for 30 minutes, followed by a 30 minute (KDR and VEGF-C stainings) or 45-minute (all other stainings) incubation with peroxidase-labelled biotin avidin complex (Vectastain). Subsequently, the sections stained with KDR and VEGF-C antibodies were incubated for 10 minutes with biotinylated tyramine (dilution 1:200), followed by a 20-minute incubation with ABC-peroxidase solution (catalysed reporter deposition method [36]). All stainings were developed by a 10-minute incubation with a 0.4 mg/ml 3-amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany).

Four micron paraffin-embedded serial sections were deparaffinized in xylene and rehydrated in an ethanol series. Antigen retrieval was performed by boiling sections for 10 minutes in sodium citrate buffer (pH=6.0) using a microwave oven. Endogenous peroxidase activity was blocked by treating the sections for 30 minutes with PBS containing 3% hydrogen peroxide. Then, sections were

Figure 1. PAS-histochemistry (**a**) and immunohistochemical analysis of CD34 (**b**) and EMAP-II expression (**c**,**d**) and presence of macrophages (i.e. CD68-positive cells) (**e**,**f** (KP1), **g** (MG-P1)) and laminin (**h**) in (serial) sections of primary uveal melanoma. PAS-histochemistry without bleaching decorates loops and network patterns containing melanophages (arrowhead, **a**). The vasculature was identified by an anti-CD34 mAb (**b**), often the endothelial lining appeared to be discontinues (arrowhead) and damaged (arrow). In areas with strongest EMAP-II immunoreaction (**c**), abundant CD68 staining is present associated with the vasculature (CD34 staining of the boxed area is depicted in **b**), indicating the accumulation of macrophages (**e**). Single asterisks indicate these corresponding areas (**c**,**e**). EMAP-II positivity of tumor cells in area without accumulation of macrophages can be explained by the detection of its precursor p43 which is retained intracellularly.[17,35] EMAP-II positivity of tumor cells in area without accumulation of macrophages is explained by the detection of the EMAP-II 34,000 form which is retained intracellulary.[17,35] Inflammatory infiltrating cells (other than macrophages) are negative for EMAP-II (**c** **). Especially in melanoma containing loops and network patterns (arrow, **d**), EMAP-II immunoreaction was high (**d**). Next to macrophages (arrow, **f**), tumor cells were occasionally positive for CD68 stained by the KP1 mAb (*, **f**). In a serial section macrophages are stained for CD68 by MP-G1 mAb (**g**) showed that they are closely related to the extracellular matrix structures containing laminin (**g**,**h**). By using CD68 mAb Kp1 and MG-P1, we confirmed earlier data of Mäkitie et al.[6] Stainings in **b**-**h** were counterstained with Harris' haematoxylin. Magnification **a**,**b**,**d**,**f**: 100x. **c**,**e**: 40x, **g**,**h:** 400x

incubated with 20% normal horse serum (Vector Laboraties) for 10 minutes. Of each specimen, the sections directly adjacent to the section stained by Azan histochemistry were incubated with QBEnd/10 mAb (diluted: 1:50) and further processed as described above.

In control sections, primary antibodies were omitted. All stainings were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature and were mounted in Imsol-mount medium (Klinipath B.V., Duiven, The Netherlands).

Determination of macrophage density

In a subset of tumors expressing EMAP-II, the density of macrophages in areas with low or without immunoreaction and in areas with strong immunoreaction was assessed by visual examination of EMAP-II and CD68 immunostainings using an ocular with counting grid at 163x magnification. In a tumor containing areas with both low and with strong EMAP-II immunoreaction, numbers of macrophages were counted in 10 non-overlapping fields for either level of immunoreaction. Statistical analysis was performed by a paired t-test.

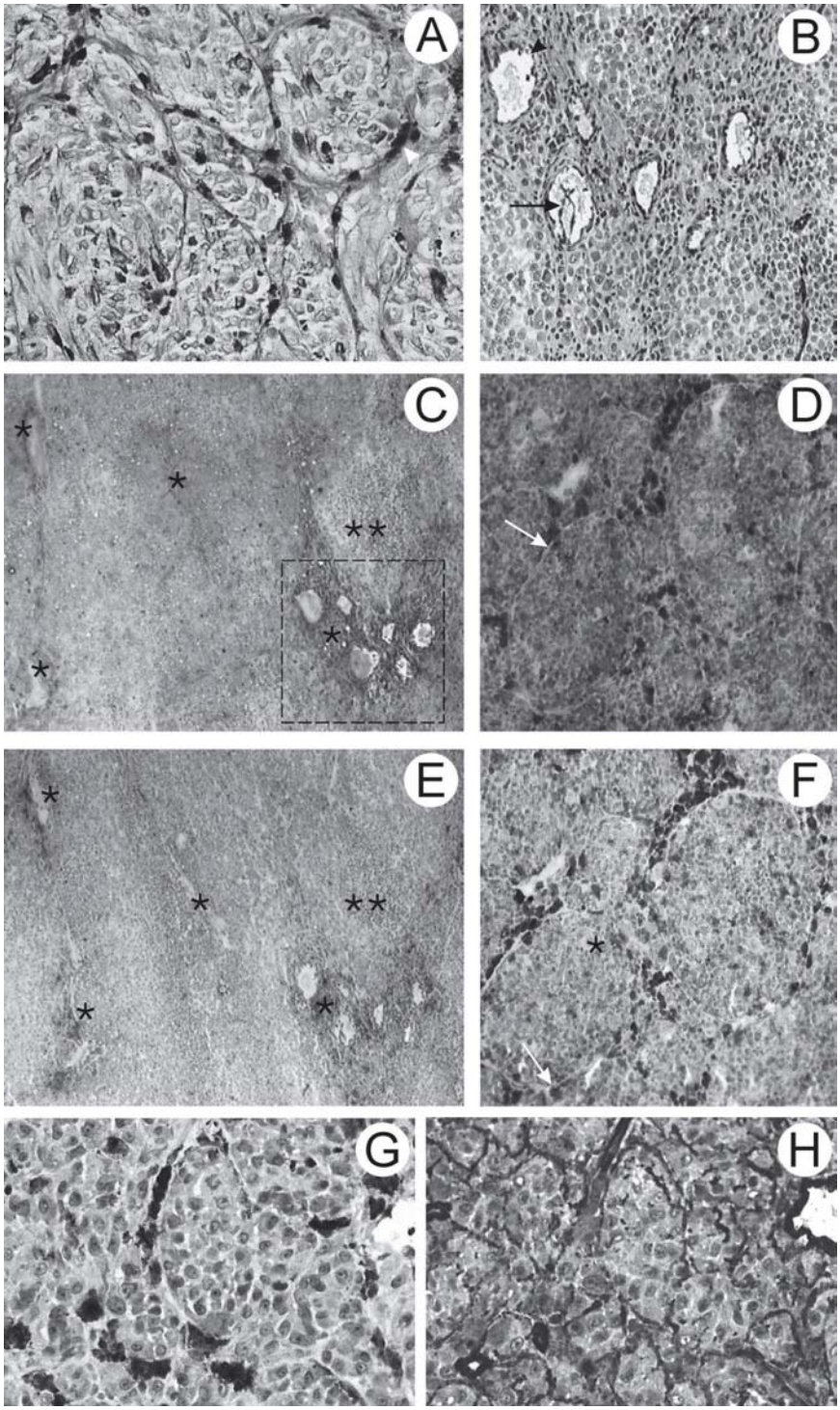
Detection of apoptosis

Apoptotic cells were detected in four micron cryosections using the ApopTag Kit S7100 from Introgen Therapeutics, Houston, Texas, USA, according to the manufacturer's guidelines.

RESULTS

Vascular staining

In all 25 uveal melanomas, the vasculature was identified by anti-CD34 mAb staining on frozen sections (Table I) and morphological properties as described previously.[5,13,37] Often, the endothelial layer was discontinuous, compatible



with endothelial damage (figure 1b). Although blood vessel density varied among different lesions, no evident increase of blood vessel density in distinct areas within a tumor lesion was observed. Azan and PAS histochemistry (figure 1a) and CD34-immunohistochemistry of serial paraffin-embedded sections indicated that the blood vasculature was closely associated with loops and network patterns as described previously.[5]

Co-localization of EMAP-II expression and macrophages

EMAP-II expression was detected in 23 out of 25 (92%) uveal melanomas as a cytoplasmic staining of tumor cells (figure 1c,d). In 15 tumors, EMAP-II expression was localized in circumscript areas. Especially tumor cells directly surrounding blood vessels were strongly positive for EMAP-II. In 8 melanomas, all tumor cells were intensely positive for EMAP-II (referred to as ubiquitous immunoreaction). By comparison to parallel endothelial CD34 staining in serial sections, EMAP-II was also present on blood vascular endothelium in 3 tumors in the areas with local expression of EMAP-II, whereas in 6 tumors with ubiquitous EMAP-II staining of tumor cells, endothelial cells were also ubiquitously positive. Especially in areas of loops and network patterns, all tumor cells showed strong EMAP-II immunoreaction (figure 1d). Strong endothelial EMAP-II immunoreaction was also observed in areas with necrosis (not shown). Finally, background staining patterns that could interfere with EMAP-II expression analysis were not observed when using other polyclonal antibodies (e.g. anti-vWF polyclonal antibodies).

In all 25 melanomas, macrophages could be detected by CD68 staining and by morphological characteristics (compare serial sections in figures 1e and f). When using Kp1 antibody, tumor cells were also weakly positive for CD68 in 8 melanoma lesions (figure 1f), whereas incubation with the PG-M1 mAb did not result in CD68 staining of melanoma cells (figure 1h). These data confirm earlier results.[6,38] Both macrophages and melanophages were strongly associated with the extracellular loops and network patterns which contain laminin (figure 1g,h).[5]

In the 15 tumors with local differences in EMAP-II staining intensity, macrophages were consistently more abundant in areas with higher EMAP-II levels, especially around blood vessels (compare serial sections in figures 1c and e). Counting of the numbers of macrophages in 4 of these tumors demonstrated a significant difference ($p<0.0001$) between those in tumor areas with negative to low EMAP-II immunoreaction (72±49 (mean±SD) macrophages/mm^2) and those in areas with strong EMAP-II immunoreaction (457±129 macrophages/mm^2). Macrophages were detected around both EMAP-II negative (n=12) and positive (n=3) blood vessel endothelium. In 8 melanomas with ubiquitous EMAP-II immunoreaction, macrophages were equally distributed over the tumor tissue. In these tumors, macrophages were present around EMAP-II negative and positive (n=7) vessels as well. In areas containing loops and network extracellular matrix patterns, nests of tumor cells were intensively positive for EMAP-II immunostaining. In addition, EMAP-II immunoreaction was lower in areas containing no loops and network patterns compared to areas with these patterns within one lesion.

ICAM-1 and VCAM-1 immunostaining

Co-expression of ICAM-1 and EMAP-II was found on tumor cells in all 23 EMAP-II positive cases (figure 2a,b). Conversely, in areas with no or low tumoral EMAP-II immunoreaction, ICAM-1 could not be detected on tumor cells. In only 3 out of 15 tumors with local EMAP-II expression, ICAM-1 expression was observed on EMAP-II positive endothelium whereas this was the case in 6 out of 8 tumors (figure 2a,b) with generalised immunoreaction. ICAM-1 was often expressed on a subset of endothelial cells (figure 2c,d). In this group of

tumors, EMAP-II negative endothelium did not express ICAM-1 (central vessel in figure 2a,b).

In only 4 melanomas, delicate VCAM-1 staining could be detected on both EMAP-II positive and negative blood vascular endothelium (not shown). VCAM-1 expression on tumor cells was never observed.

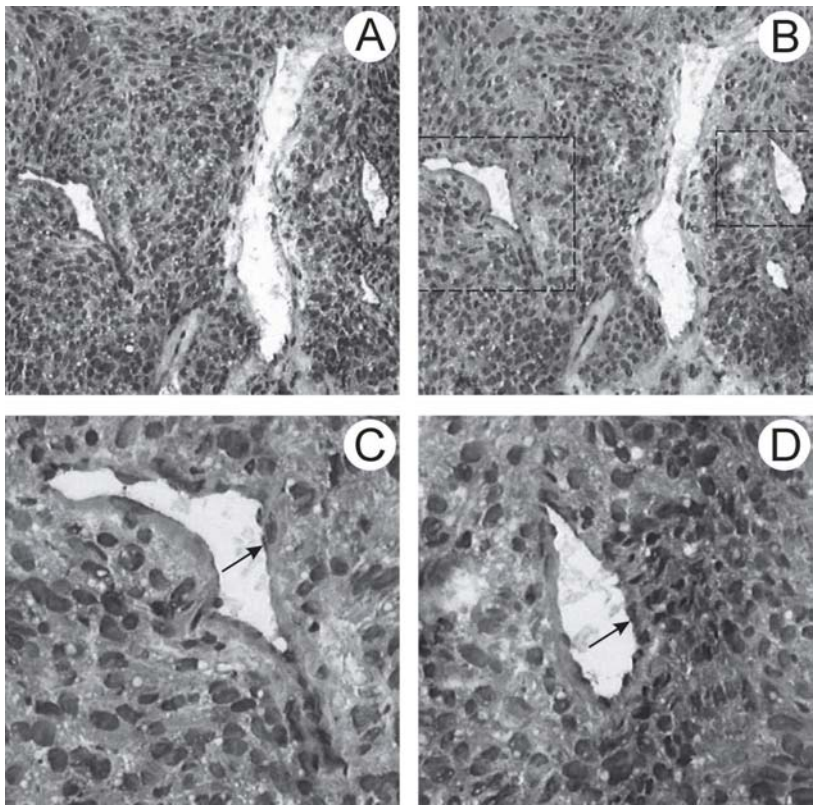


Figure 2. Immunohistochemical analysis in serial sections of EMAP-II (**a**) and ICAM-1 (**b**) expression in primary uveal melanoma (magnification: 100x). Intense ubiquitous EMAP-II staining and ICAM-1 expression are observed at both tumor and endothelial cells (**a**,**b**). Insets in **b** are shown enlarged in **c** and **d** (magnification: 250x). Part of the EMAP-II positive endothelial cells express ICAM-1 (arrow **c**,**d**) whereas the central EMAP-II negative vessel expressed no ICAM-1 (**a**,**b**). All stainings were counterstained with Harris' haematoxylin.

KDR and vWF immunostainings

We analyzed 16 EMAP-II-positive uveal melanomas for the presence of KDR and vWF as markers of endothelial activation and damage, respectively. In 14 melanomas, comparison to parallel EMAP-II staining demonstrated that EMAP-II positive endothelial cells lacked expression of KDR (figure 3a,b). However, strong staining of vWF in endothelial cells and underlying tissue indicated a release of vWF by this endothelium (figure 3a,c). Conversely, EMAP-II negative endothelial cells did express KDR in 5 melanomas (figure 3d,e) whereas no evident damage could be detected in these cells by vWF staining (not shown). In only 2 tumors a weak co-localization of EMAP-II positivity, KDR and absence of vWF-release was observed. Note that patterns similar to patterns that of EMAP-II staining were not observed when using other polyclonal antibodies (e.g. anti-vWF polyclonal antibodies).

Detection of apoptosis

Apoptosis was detectable in only 3 out of 15 uveal melanomas. In none of the tumors, apoptotic endothelial cells could be found. In one tumor, an evidently necrotic area, positive for EMAP-II, showed staining whereas in the remaining tumor cells apoptosis was absent. In the other 2 tumors, apoptosis was confined to a few tumor cells dispersed over the entire tumor area (not shown).

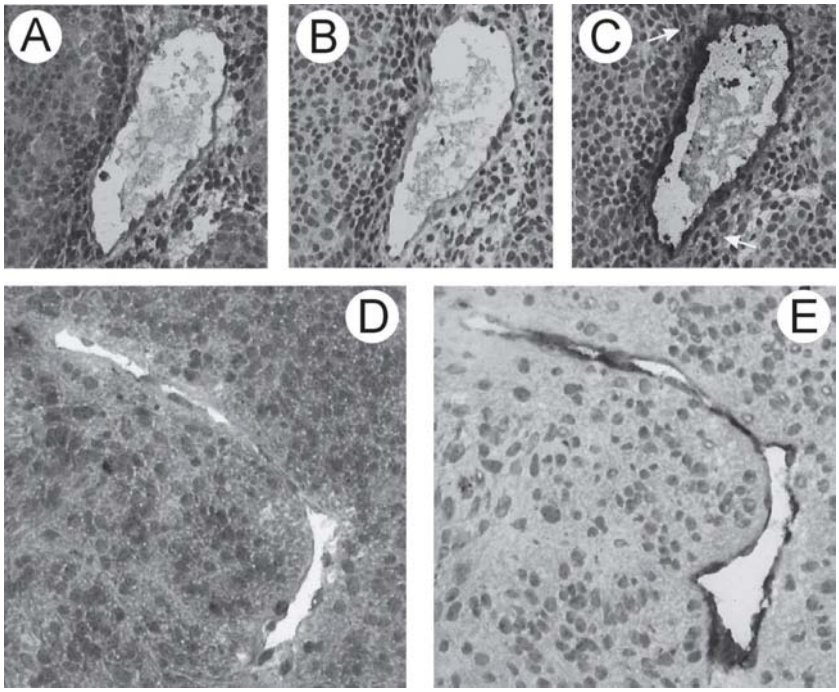


Figure 3. Immuno-histochemical analysis in serial sections of EMAP-II (**a**,**d**), KDR (**b**,**e**) and vWF (**c**) expression in primary uveal melanoma. Although endothelial EMAP-II immuno-reaction is delicate (**a**), KDR is not expressed (**b**) whereas endothelial damage is shown by leakage of vWF towards surroun-ding tumor tissue (arrows, **c**). In the absence of endothelial EMAP-II expression (**d**), KDR is expressed (**e**). All stainings were counterstained with Harris' haematoxylin. Magnification: 250x

Absence of co-localization of VEGF-C expression and macrophages

Finally, we analyzed 12 melanomas for VEGF-C expression. In 6 melanoma, VEGF-C was expressed in restricted areas. No evident co-localization of accumulation or absence of macrophages and VEGF-C expression was observed in any lesion (data not shown).

Correlation with clinical outcome:

In only one patient, liver metastasis was found 2 years after enucleation. This tumor contained PAS-positive loops and network patterns, ubiquitous EMAP-II immunoreaction of both tumor and endothelial cells and macrophages, was equally distributed over the lesion. In addition, ICAM-1 was expressed by the tumor and a subset of endothelial cells. No VCAM-1 expression or apoptosis were observed. EMAP-II positive endothelial cells lacked expression of KDR but strongly stained for vWF. VEGF-C expression was not evaluated.

DISCUSSION

Recently, we described that the arcs, loops and network extracellular matrix patterns may represent a fluid-conducting meshwork in xenografted and primary uveal melanoma.[5] Our findings indicated that these structures closely parallel the previously described vascular channels.[39] Although it has been demonstrated that melanoma cells may express non-melanocytic markers like endothelial cell-associated CD34 and macrophage-associated CD68,[39,40] we could not confirm the presence of blood-conducting channels lined by tumor cells. Instead, septa consisting of extracellular matrix components that could be visualized by PAS and Azan histochemistry, surrounded tumor cell nests. Remarkably, many macrophages (i.e. CD68 positive cells) were found associated with this meshwork in melanoma xenografts. We now have studied the presence and localization of macrophages in uveal melanoma, focusing on

the mechanisms underlying infiltration by this cell type. Our results also show that in uveal melanoma, macrophages are abundantly present and co-localize with the extracellular matrix patterns containing laminin.[5,41]

In uveal melanoma containing loops and network extracellular matrix PAS-positive patterns, the monocyte chemoattractant protein EMAP-II was abundantly present in the cytoplasm of tumor cells, which was accompanied by local macrophage accumulation. Additional analysis in uveal melanomas lacking these patterns showed that different levels of local EMAP-II staining within one tumor were present and that macrophages were preferentially located at areas with highest EMAP-II immunoreaction. These data supported the hypothesis that tumor cells recruit local resident tissue macrophages and peripheral monocytes by release of EMAP-II. This hypothesis was corroborated by several previous studies on the expression of EMAP-II in tumor cells and the ability of EMAP-II to attract macrophages.[14,15] Indeed, infiltration of the tumor may be facilitated by the loops and network patterns by serving as a gateway. However, secondary changes including extensive retinal detachments, glaucoma, rubeosis and intraocular hemorrhage may contribute to the influx of macrophages as well since the majority of the lesions analyzed were large. Since VEGF-C expression did not co-localize with absence or accumulation of macrophages, our data suggest that VEGF-C does not play a substantial role in macrophage invasion in uveal melanoma.

EMAP-II is normally retained intracellulary and can be released and partially processed by several triggers, including apoptosis and hypoxia.[17,20] Since apoptotic cells were hardly present in the tumor lesions, this does not seem to be a decisive factor in EMAP-II expression in uveal melanoma. Hypoxic tumor cells may express hypoxia inducible factor -1 (HIF-1) [8,42] and release vascular endothelial growth factor-A (VEGF-A). In this respect, it is noteworthy that we observed no release of VEGF-A in these primary uveal melanoma lesions.[13] However, to the best of our knowledge, expression of HIF-1 in uveal melanoma has never been reported thus far.

Purified recombinant mature EMAP-II protein is able to activate endothelial cells and enhance the expression of adhesion molecules P- and E-selectin.[14] These adhesion molecules mediate invasion of different types of leukocytes into the underlying tissue (for review see ref. 27). Macrophage infiltration is primarily mediated by ICAM-1 and VCAM-1 adhesion molecules, however. Remarkably, one of our first observations was that EMAP-II and ICAM-1 are co-expressed on tumor cells. This indicates that either both these molecules are upregulated by the same stimulus, or that EMAP-II expression induces ICAM-1 expression by an autocrine mechanism. Moreover, ICAM-1 was predominantly expressed on the vascular endothelium in those tumors that showed intense ubiquitous staining for EMAP-II. In tumors with focal EMAP-II immunoreaction, the endothelium was often negative for both EMAP-II and ICAM-1, suggesting that a certain threshold level of EMAP-II binding to endothelial cells is necessary for ICAM-1 induction. Functional experiments in cell cultures are necessary to confirm these findings. Since only 4 tumors expressed VCAM on their vessels, it is unlikely that this adhesion molecule is induced by EMAP-II expression and contributes to infiltration of monocytes.[27]

In order to further elucidate the role of endogenously produced EMAP-II in primary uveal melanoma, we evaluated additional parameters that have been associated with EMAP-II expression in the literature, i.e. the induction of

apoptosis and of angiogenesis. As stated earlier, EMAP-II is involved in macrophage influx in several types of carcinomas. This has been related to tumor progression [9,10] and may partly be explained by macrophage-induced neovascularization [43] (for review see ref. 44). We hypothesized that in primary uveal melanoma, hypoxic tumor cells secrete EMAP-II contributing to recruitment and infiltration of monocytes into the tumor tissue. Our results suggest that this sequence of events is not followed by angiogenesis: EMAP-II positive endothelium did not express KDR as a sign of endothelial activation like occurring during angiogenesis. Rather, our data suggest extensive release or leakage of vWF out of the EMAP-positive endothelial cells into surrounding tumor tissue, indicating endothelial cell damage. These findings imply that EMAP-II, instead of triggering angiogenesis, rather induces endothelial cell death *in vivo*. In uveal melanoma therefore, EMAP-II appears to have an adverse effect on the neovasculature, supporting the results obtained by administering exogenous EMAP-II in previous studies.[14,18,45] It was demonstrated that EMAP-II did induce endothelial apoptosis [18] after release by apoptotic (tumor) cells. However, in our study no evident apoptosis of EMAP-II positive endothelial or tumor cells was observed. The involvement of apoptosis, either as a trigger of EMAP-II induction or as a result of its formation, remains therefore questionable, at least in uveal melanoma. Our data support earlier results indicating that processing and release of EMAP-II is not restricted to apoptotic cells but may also occur in cells that ultimately die by ischemia and subsequent necrosis.

The question arises whether local administration of EMAP-II will induce extensive vascular damage which may lead to necrosis of the tumor. Previous data of our laboratory indicated that the PAS-positive loops and network patterns may be involved in tumor cell nutrition (chapter 6). So, by inhibiting angiogenesis via EMAP-II administration, uveal melanoma may (partly) escape this intervention by formation of loops and network patterns. Local administration of EMAP-II may also lead to extensive influx of macrophages which may trigger an immune response or, alternatively, result in release of growth factors necessary for tumor growth and subsequent metastasis. Thus, the effects of EMAP-II expression are complex, the possible effects of intervention with EMAP-II expression in uveal melanoma or other tumor types are yet unpredictable and, therefore, require extensive additional research.

Data of clinical outcome of 16 patients could be obtained with only in one patient proven metastatic disease. This could be explained by the relatively short follow-up time in the primary uveal melanoma patients, since this tumor is notorious of metastatic disease after years. Therefore, our study does not allow to draw any conclusion on the role EMAP-II of expression in determining prognosis.

In summary, our data are in line with the following sequence of events: tumor cells promote adhesion and invasion of macrophages via the release of EMAP-II. This process is mediated by EMAP-II-induced expression of ICAM-1 on endothelial cells. Infiltrating macrophages follow the PAS-positive extracellular matrix patterns. Binding of substantial amounts of EMAP-II to vascular endothelial cells may lead to endothelial damage, resulting in vWF release.

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Summarizing discussion

Samenvatting

Adapted from "Pathophysiological implications of stroma pattern formation in uveal melanoma"

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Malignant solid tumors consist of a mixture of neoplastic and non-neoplastic (host) cells. In this respect, a solid tumor can be regarded as a functional tissue in which architecture and function are maintained by a dynamic interplay between tumor cells and a microenvironment consisting of extracellular matrix containing fibroblasts, blood and lymphatic vasculature and infiltrating and resident immune cells (i.e. tumor stroma).[1] Indeed, evidence is accumulating that many stromal components are able to activate cellular processes as growth, death, adhesion, migration, invasion, gene expression and differentiation.[2-5] Therefore, the involvement of tumor stroma appears not to be restricted to deposition of structural components only, but also includes release of cytokines, chemokines and growth factors by tumor and host cells and regulation of their final effects. The importance of understanding the role of extracellular matrix (ECM) and interaction of tumor and host cells has been further emphasized by studies showing that deposition of extracellular matrix and its interactions are of essential importance for primary tumor growth and rate of metastasis (reviewed in refs. 6-8), which is the major cause of death in cancer patients. In this thesis, we have studied the role of the ECM compartment with respect to tumor growth, progression and metastasis. In more detail, in chapter 2, 3 and 4, we studied the lymphatic and blood vasculature and the key players in the process of lymph- and hemangiogenesis. In chapter 5, 6 and 7, we studied ECM deposition patterns present in cutaneous and uveal melanoma which have been associated with poor prognosis.[9-12]

ECM: lymphatic and blood vasculature

The significance of the ECM for tumor metastasis may be explained by the close relationship of tumor cells with the lymphatic and blood vasculature in this compartment. The exact anatomical relation between tumor cells and the lymphatic bed is unclear since reliable lymphatic endothelial markers are lacking.[13] Recently, however, a few promising lymphatic markers have been identified [14-16] and, in addition, gene array profiling data identified several new genes with specific expression in lymphatic endothelial cells.[17] These latter data would facilitate the possible identification of new additional markers. To be able to study the role of the lymphatic vasculature in tumor growth and metastasis in our material, we further explored differential staining of the blood and lymphatic vasculature by a previously described double staining protocol using the blood endothelial marker PAL-E and pan-endothelial marker CD31 (chapter 2, 3 and 4).[18] We showed that the lymphatic vasculature could reliable be detected in different types of normal tissues and malignant tumors, being confined to the peritumoral stroma (kidney and liver carcinoma lesions) and in the stroma between tumor cell nests (melanoma, breast, colon and larynx carcinoma lesions) (chapter 2-3). These data are in line with previous studies.[18-22]

Metastasis to regional lymph nodes may be mediated by pre-existent or, perhaps, newly formed lymphatics (i.e. via tumor-induced lymphangiogenesis). Tumor-induced lymphangiogenesis has been reported in xenografted tumors which overexpressed the lymphatic growth factors VEGF-C and D (vascular endothelial growth factor-C, -D [23-26]).[25,27-31] In addition, few studies suggested the existence of tumor-induced lymphangiogenesis in human cancer.[20,22] Furthermore, its existence is supported by the relationship between expression

of VEGF-C and lymph node metastasis in different human tumor types.[32-38] However, we (chapter 2, 3 and 4) and others [18,19,39] did not observe evident signs of lymphangiogenesis in a panel of different tumor types. As explained in chapter 1 (figure 1, page 14), tumor-induced lymphangiogenesis should involve binding and activation of Flt-4, present on the lymphatic endothelium [40] by VEGF-C and VEGF-D.[23-25,25-31,41] However, VEGF-C and Flt-4 are both expressed in uveal melanoma lesions, a tumor type lacking lymphatics and consequently lymphangiogenesis (chapter 4). Therefore, these data indicate that the concerted action of VEGF-C and Flt-4 is not sufficient for lymphangiogenesis to occur in, at least, uveal melanoma. Indeed, tumor-induced lymphangiogenesis, if it exists, may rather be a sporadic than a general phenomenon in human cancer.[42] Furthermore, it remains to be determined whether these new lymphatics are functional.[43]

In normal adult tissues, Flt-4 expression is restricted to the lymphatic endothelium.[23,40] However, Flt-4 is also expressed on intra- and periumoral blood vessels in different types of malignant tumor lesions (chapter 2, 3 and 4).[21,44] which may be explained by tumor cell release of VEGF-A (a major angiogenic growth factor) (chapter 3). In addition, other factors, like VEGF-C, may also be involved (chapter 4). Next to lymphangiogenesis, Flt-4 and its ligand VEGF-C have been involved in tumor-induced angiogenesis.[45-47] Therefore, if induction of Flt-4 expression on tumor blood vessels would be a general phenomenon, Flt-4 would be a marker for tumor endothelium and, hence, it may serve as a therapeutic target. By targeting Flt-4, blood vessels in the tumor area would selectively be targeted and the formation of tumor-associated lymphatics may be inhibited leading ultimately to reduction of both tumor growth and metastasis.[43,48,49] Our data (chapter 2) also suggested that Flt-4 expression on blood vessels gradually increases as a tumor becomes more malignant in case melanocytic skin lesions. Indeed, besides providing a possible therapeutic target, immunohistochemical analysis of Flt-4 expression may provide additional support in determining clinical outcome.

ECM formation in uveal melanoma

Although it is known that the ECM contains blood vessels, the exact nature and function of the depositions in uveal melanoma have not been elucidated yet.[11,12,50-54] Relevant research started in the early nineties when Folberg et al. proposed nine distinct morphological types of microvessel architecture in uveal melanoma.[11] These microvascular patterns were identified in tissue sections by fluorescein-conjugated Ulex europaeus I and by periodic acid-Schiff (PAS) histochemistry that stains basement membrane and collagen.[11,12] In addition, we showed that these PAS-positive patterns could also be detected by Azan and laminin (immuno)histochemistry in primary uveal and xenografted melanoma (chapter 4 and 5) The PAS-positive patterns, arranged in arcs, loops and networks, proved to be related to rate of metastasis and, hence, prognosis.[11,12] Since then, similar patterns in human metastatic cutaneous melanoma, breast, ovarian and prostate carcinoma could be related to prognosis.[9,10,55-57]

Re-assessment by an additional study questioned the true microvascular nature of the patterns [50] as Foss et al. showed that the patterns consisted of extracellular matrix in which blood vessels were embedded, the so-called "fibrovascular tissue". These results were supported by data presented in

chapter 5 and 6, suggesting that PAS-positive structures surrounding tumor cell nests represented fibrovascular septa. These may act as a matrix network that provides a medium for diffusion of soluble factors (i.e. a fluid-conducting meshwork). Finally, Maniotis et al.[51] suggested that vascular channels were closely associated with the PAS-positive arcs, loops and network patterns. Vascular channels were defined as blood-conducting channels lined by tumor cells (and therefore not by endothelium) in close contact with the blood vasculature. In these channels along the PAS-positive patterns, melanoma cells mimic endothelial cells (i.e. vasculogenic mimicry [51,52]). Since the first report of the possible existence of vascular mimicry, a number of proteins involved in the formation of the PAS-positive arcs, loops and network patterns and in vasculogenic mimicry have been identified.[58-61] In addition to the possible phenomenon of melanoma cells mimicking endothelial cells, it was suggested that these tumor cells may also express cell-fate determination molecules which are normally expressed during embryonic vasculogenesis, and may participate in neovascularization in ischemic muscle.[62,63] Besides channels lined by tumor cells, also "mosaic" vessels in which endothelial cells and tumor cells alternatingly form the luminal surface, may be present in tumors.[64]

The existence of vasculogenic mimicry was seriously questioned since several authors, including us, did not observe conductance of blood along the PAS-positive structures outside the blood vasculature.[53,54] In more detail, by applying Azan histochemistry resulting in a sharp contrast of red-stained erythrocytes and blue-stained matrix components, combined with immunohistochemical analysis using a panel of established endothelial markers, no evident presence of endothelium-free blood-conducting structures were observed (chapter 5) although it was found that melanoma cells may express endothelial markers as well.[65] So, reliable identification of blood-conducting structures was questioned (for review see ref. 54), thereby hampering a definitive elucidation whether vasculogenic mimicry is present or absent in malignant tumors.

ECM patterns: morphological and functional properties

As stated above, Foss et al. proposed that the PAS-positive arcs, loops and network patterns constitute fibrovascular tissue,[50] which was confirmed by us using electron microscopy (chapter 5). In addition, our observations suggested that extracellular matrix is deposited as curved sheets around nests or nodules of tumor cells. In these structures, blood vessels are embedded and, at least in uveal melanoma, lymphatics are absent (chapter 4). The extracellular matrix pattern is present between spheres of tumor cells and is probably deposited as envelopes. In 2D-analysis, this matrix deposition is detected as arcs, loops and network patterns. Intravenous injection of fluorescein isothiocyanate-labeled bovine serum albumin in nude mice bearing subcutaneous xenografted melanoma containing the arcs, loops and network patterns showed that this tracer was able to leave the blood stream and enter the arcs, loops and network patterns (chapter 5).[66] This observation suggested that exudate-like fluid is transported (conducted) by diffusion and/or by a fluid stream via extracellular spaces located in the patterns. Therefore, we proposed in chapter 5 and 6 that the arcs, loops and network patterns represent a "fluid-conducting meshwork". In this respect, it is noteworthy that the original reporters of the existence of vasculogenic mimicry, Maniotis et al.,[51] suggested recently the

presence of plasma within laminin-positive looped patterns from contiguous leaky vessels in melanoma.[67] Indeed, in our opinion, the term "channel" as description of fluid-conducting extracellular spaces may be incorrect since the concept of blood conducting channel formation by tumor cells postulated by Maniotis et al.[51] is questionable.

In primary uveal melanoma containing arcs, loops and network patterns, microvascular density is often so low that necrosis is to be expected.[52] However, necrotic foci are absent in these areas.[52] Previously, it was suggested that this phenomenon could be explained by the presence of vascular channels.[51,52] However, our data suggest that vascular channels (if they exist) may not be the only contributor involved since exudate-like fluid is transported along the extracellular matrix patterns as well (chapter 5 and 6). Indeed, small molecules like most nutrients are able to reach tumor cells located at some distance from the blood vasculature within 2 minutes (chapter 6). In addition, besides nutrition of tumor cells, uveal melanoma could also acquire an alternative system to drain excess tissue fluids by formation of extracellular matrix networks, as a substitute for a lymphatic system which is absent in this type of tumor (chapter 4).

ECM matrix patterns: tumor and associated host cells

The arcs, loops and network extracellular matrix patterns harbor different types of cells, including endothelial cells, pericytes, fibroblasts and inflammatory cells including macrophages (chapter 5, 6 and 7).[68] Although it is unknown how the patterns are formed, the presence of fibroblasts suggested their involvement. Matrix may be deposited by stromal cells, melanoma cells or both. In any case, they seem to be induced primarily by the presence of the tumor cells, and are therefore fundamentally different from a pre-existent stroma in which a tumor lesion invades.

The abundant presence of macrophages along the arcs, loops and network patterns in uveal melanoma (chapter 5 and 7) [68,69] supports the idea that migratory cells leave tumor vessels and invade by adhering to matrix components present in stromal sheets, thereby migrating actively into the spaces between them or drilling their way by proteolytic enzymes expressed at their migratory front. Otherwise, it is possible that the extracellular matrix patterns may also serve as a gateway for tumor cells to escape from the tumor, explaining thereby the higher degree of malignancy.[9-12,55-57] The exact function of macrophages in tumor growth and metastasis is essentially unknown, although macrophages have been associated with a poor prognosis in different tumor types (for review see refs. 70) including uveal melanoma.[69,71-74] Indeed, the number of macrophages is related to microvascular density (for review see ref. 75), indicating that they induce neovascularization [76] which is essential for tumor growth and metastasis.[77] In addition, their strict colocalization with the arcs, loops and network patterns (chapter 5 and 7) [68,69] suggests also a role in the development of these patterns. It is largely unknown how invasion of macrophages into the arcs, loops and network patterns is mediated, although expression of chemokines like monocyte chemotactic protein-1 [78] and endothelial-monocyte activating polypeptide-II (chapter 7) [79,80] by melanoma cells has been identified. EMAP-II expression was closely associated with macrophages distributed along the arcs,

loops and network patterns, suggesting that EMAP-II may act as chemotactic factors for the tumor-infiltrating cells.

In conclusion, in this thesis, we provided evidence suggesting that the ECM compartment of solid tumors is of essential importance for tumor growth and metastasis. Indeed, we showed that it represents the tumor compartment in which tumor cells interact with the blood and lymphatic vasculature. Furthermore, the formation of the arcs, loops and network ECM patterns may 1) accommodate transport of plasma-derived molecules (nutrients) into the tumor lesion and waste products away from the tumor tissue, 2) serve as an alternative lymphatic system regulating interstitial pressure, 3) mediate infiltration of tumor tissue by host-derived cells and 4) provide a gateway for tumor cells to leave the tumor.

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Samenvatting

Het melanoom is een kwaadaardige tumor die uitgaat van pigmentvormende cellen (melanocyten) die zich voornamelijk in de huid en het oog bevinden. Het huidmelanoom is een van de meest voorkomende kwaadaardige tumoren bij jonge volwassen (in Nederland ca 2000 nieuwe gevallen per jaar). Het oogmelanoom is de meest frequente oogtumor. Deze laatst genoemde tumor wordt per jaar bij iets meer dan 100 mensen in Nederland vastgesteld. Beide tumortypen hebben sterk de neiging tot uitzaaien (metastasering), waarbij het oogmelanoom een sterke voorkeur heeft voor de lever. Eenmaal gemetastaseerd, is de prognose van beide typen melanomen slecht.

Behalve cellen wordt in zowel huid- als oogmelanomen vaak tussencelstof (oftewel extracellulaire matrix (ECM)) aangetroffen. Eigenschappen van deze ECM blijken essentieel te zijn voor het optreden van metastasering. In de ECM bevinden zich ook andere celtypen, zoals ontstekingscellen, cellen die ECM produceren (fibroblasten), bloed- en lymfevaten. Tumoren zijn sterk afhankelijk van deze bloedvaten om te kunnen uitgroeien: zonder bloedvaten kunnen tumoren niet groter worden dan circa 2 mm. Om te kunnen groeien, produceren tumorcellen dan ook groeifactoren die vorming van nieuwe bloedvaten (angiogenese) in en direct rond de tumor induceren. Een van de meest belangrijke groeifactoren is vasculair endotheel groeifactor (VEGF).

Via de bloedbaan kunnen tumorcellen zich verplaatsen naar, en uitgroeien in, weefsels en organen elders in het lichaam (metastasering op afstand). Naast bloedvaten, zijn ook lymfevaten betrokken bij het metastaseringsproces, namelijk naar lymfeklieren die nabij de tumor zijn gelokaliseerd (regionale metastasering). In muizenstudies zijn er reeds duidelijke aanwijzingen gevonden dat tumorcellen ook bepaalde groeifactoren (zoals VEGF-C en VEGF-D) kunnen aanmaken die de uitgroei van nieuwe lymfevaten (lymfangiogenese) in de tumor (en daarmee de lymfogene metastasering) kunnen stimuleren. Of lymfangiogenese ook in humane tumoren voorkomt is echter nog onduidelijk.

Nieuwvorming van bloed- en lymfevaten is het gevolg van de interactie tussen de tumorcellen en de endotheelcellen die de bloed- en/of lymfevaten bekleden. Inmiddels is er steeds meer wetenschappelijk bewijs dat er tussen de tumorcellen en aanwezige andere celtypen inderdaad talrijke onderlinge interacties plaats vinden. Deze interacties worden bewerkstelligd door verschillende soorten ‘signaalstoffen’ (zoals VEGF, VEGF-C en VEGF-D) die zowel door de tumorcellen en andere celtypen aangemaakt en uitgescheiden worden in de ECM.

In melanomen, zowel van de huid als het oog, zijn bepaalde patronen van ECM afzetting (depositie) aanwezig die in ongeveer 40 tot 50% van de gevallen een soort netwerk lijken te vormen. Hoewel men weet dan de aanwezigheid van deze patronen voorspellend is voor het optreden van metastasen (en dus een slechte prognose), is er weinig tot geen kennis van morfologische en functionele eigenschappen van dit netwerk.

Uit het voorafgaande blijkt dat kennis van de eigenschappen van de ECM in het melanoom noodzakelijk is om het mechanisme van tumorgroei en metastasering te begrijpen. In dit proefschrift hebben we dan ook de rol van de ECM in het huid- en oogmelanoom bestudeerd met bijzondere aandacht voor de bloed- en lymfvasculatuur, voor de belangrijke mediatoren die angiogenese en lymfangiogenese veroorzaken (hoofdstuk 2, 3 en 4) en voor de

morfologische en functionele eigenschappen van de netwerkpatronen (hoofdstuk 5, 6 en 7).

ECM: relatie tussen lymfe- en bloedvaten

Hoewel de rol van bloedvaten bij tumorgroei en metastasering uitgebreid is bestudeerd, is er weinig bekend over de rol en anatomische lokalisatie en functie van lymfevaten in tumoren. Dit wordt veroorzaakt door het ontbreken van specifieke merkstoffen voor lymfevaten. In hoofdstuk 2 hebben we een in het eigen laboratorium ontwikkelde immunohistochemische dubbelkleuring die het mogelijk maakte om bloed- en lymfevaten van elkaar te onderscheiden in verschillende soorten normale en tumor weefsels. Het bleek dat lymfevaten vooral lokaliseerd zijn aan de rand van tumoren. Overtuigende (tekenen van) lymfangiogenese hebben we echter niet gevonden. In het oogmelanoom zijn lymfevaten zelfs compleet afwezig, hoewel VEGF-C en zijn receptoren KDR en Flt-4 (waarvan Flt-4 expressie essentieel is voor lymfangiogenese) wel tot expressie kwamen (hoofdstuk 4). Deze gegevens suggereren dat er meerdere, nog niet bekende, factoren een rol spelen bij de inductie van lymfangiogenese in (humane) tumoren.

Nieuwvorming van bloed- en lymfevaten in tumoren zijn het gevolg van vele interacties tussen de tumorcellen en andere celtypen. Een voorbeeld van zo'n interactie is de inductie van Flt-4 expressie op bloedvaten in tumoren. Onder normale, niet-pathologische, omstandigheden komt Flt-4 tijdens het volwassen leven alleen tot expressie op lymfevaten. Flt-4 expressie blijkt echter te verschijnen op bloedvaten die in, of direct aan de rand van een tumor liggen (hoofdstuk 2, 3 en 4). Bij deze inductie lijkt VEGF een belangrijke rol te spelen (hoofdstuk 3). De mate van Flt-4 expressie op de bloedvaten lijkt bovendien gerelateerd te zijn aan de graad van agressiviteit, althans bij goedaardige en kwaadaardige melanocytaire huidafwijkingen (hoofdstuk 2).

ECM patronen in het oogmelanoom

Aanvankelijk in het oogmelanoom, en later ook in het huidmelanoom, werden in het begin van de jaren 90 verschillende patronen van ECM deposities beschreven. Deze patronen kunnen zichtbaar gemaakt worden m.b.v. de zogenaamde PAS-kleuring. In hoofdstuk 5 en 6 zijn deze netwerkpatronen morfologisch en functioneel gekarakteriseerd in het humane oogmelanoom en in een muizenmodel met onderhuids gelegen melanomen. De netwerkpatronen zijn opgebouwd uit ECM (hoofdzakelijk bestaand uit collageen, laminine en verschillende typen heparansulfaatproteoglycanen) met verspreid daarin ingebed bloedvaten. Intraveneuze injectie van een merkstof (gelabeld albumine) in muizen zulke melanoom lesies toonde aan dat in de tumor de merkstof de bloedvaten kon verlaten en zich via deze netwerkpatronen te verspreiden. Echter open kanalen in de netwerkpatronen waren buiten de bloedvaten niet aantoonbaar m.b.v. electronenmicroscopie. Dit patroon van merkstofverspreiding kan worden verklaard door aan te nemen dat er transport via ruimtes gelegen tussen collagene en laminine vezels optrad, als het ware door een zeef. We denken dat de ECM netwerkpatronen dus een "vloeistofgeleidend zeefnetwerk" vormen. Verdere functionele analyse toonde aan dat kleine moleculaire stoffen relatief snel werden getransporteerd door deze netwerkpatronen (hoofdstuk 6). Daarnaast zijn er aanwijzingen dat de

netwerkpatronen ook betrokken zijn bij de afvoer van (afval)stoffen (hoofdstuk 5 en 6), en daarom wat betreft functie lijken op lymfevaten.

Behalve tumorcellen zijn er langs de netwerkpatronen verschillende andere celtypen aanwezig, waaronder macrofagen (hoofdstuk 5, 6 en 7). Hun aanwezigheid die vrijwel beperkt is tot de netwerkpatronen, suggereert dat ze deze patronen gebruiken als soort geleider om de tumor binnen te komen. Het is onbekend wat de macrofagen naar de tumor doet komen. Verschillende chemotactische eiwitten kunnen hierbij een rol spelen, zoals endotheel-monocyt activerend polypeptide-II (EMAP-II). EMAP-II expressie is gerelateerd aan de aanwezigheid van macrofagen langs de netwerkpatronen in het oogmelanoom (hoofdstuk 7). Het is dus mogelijk dat de tumor macrofagen actief naar zich toetrekt door EMAP-II tot expressie te brengen. Wellicht dat dan ook tumorcellen deze patronen kunnen gebruiken om de tumor te verlaten en om via de bloed- en lymfevaten vervolgens te metastaseren.

Concluderend hebben we in dit proefschrift duidelijke aanwijzingen gevonden dat de ECM van essentieel belang is voor tumorgroei en metastasering van het huid- en oogmelanoom. Met name de interactie met bloed- en lymfevaten, de expressie van (lymf)angiogene groeifactoren en de vorming van specifieke ECM netwerkpatronen zijn belangrijk voor deze processen. Deze netwerkpatronen zijn betrokken bij het transport van voedingstoffen vanuit het bloed de tumor in, bij transport van (afval)stoffen vanuit de tumor naar de lymfe- en bloedbaan, migratie van de tumor door verschillende andere celtypen (met name macrofagen) en bij het verlaten van tumorcellen uit de tumor. Met name deze laatste drie functies zijn kenmerkend voor lymfevaten. Kortom: de ECM netwerkpatronen zijn mogelijk een functioneel alternatief voor het ontbrekende lymfevatstelsel in het oogmelanoom.

CURRICULUM VITAE

Ruud Clarijs werd geboren op 12 mei 1971 te Halsteren. HAVO en VWO werden doorlopen aan scholengemeenschap het Moller Lyceum te Bergen op Zoom. Vanaf 1990 studeerde hij Medische Biologie en vanaf 1991 Geneeskunde aan de Rijksuniversiteit Utrecht. In het kader van deze studies werd stage gelopen bij de vakgroepen Functionele Anatomie, Experimentele Cardiologie en Pathologie. Het doctoraalexamen Medische Biologie werd behaald in 1996, het artsexamen in 1998. Vanaf augustus 1998 tot juli 2002 was hij aangesteld als arts-onderzoeker op de afdeling Pathologie, UMC St Radboud te Nijmegen. Dit onderzoek, verricht onder begeleiding van Prof. Dr. D.J. Ruiter en Dr. R.M.W. de Waal, resulteerde in dit proefschrift. Vanaf juli 2002 is hij in opleiding tot patholoog (opleider Prof. Dr. D.J. Ruiter, UMC St. Radboud).

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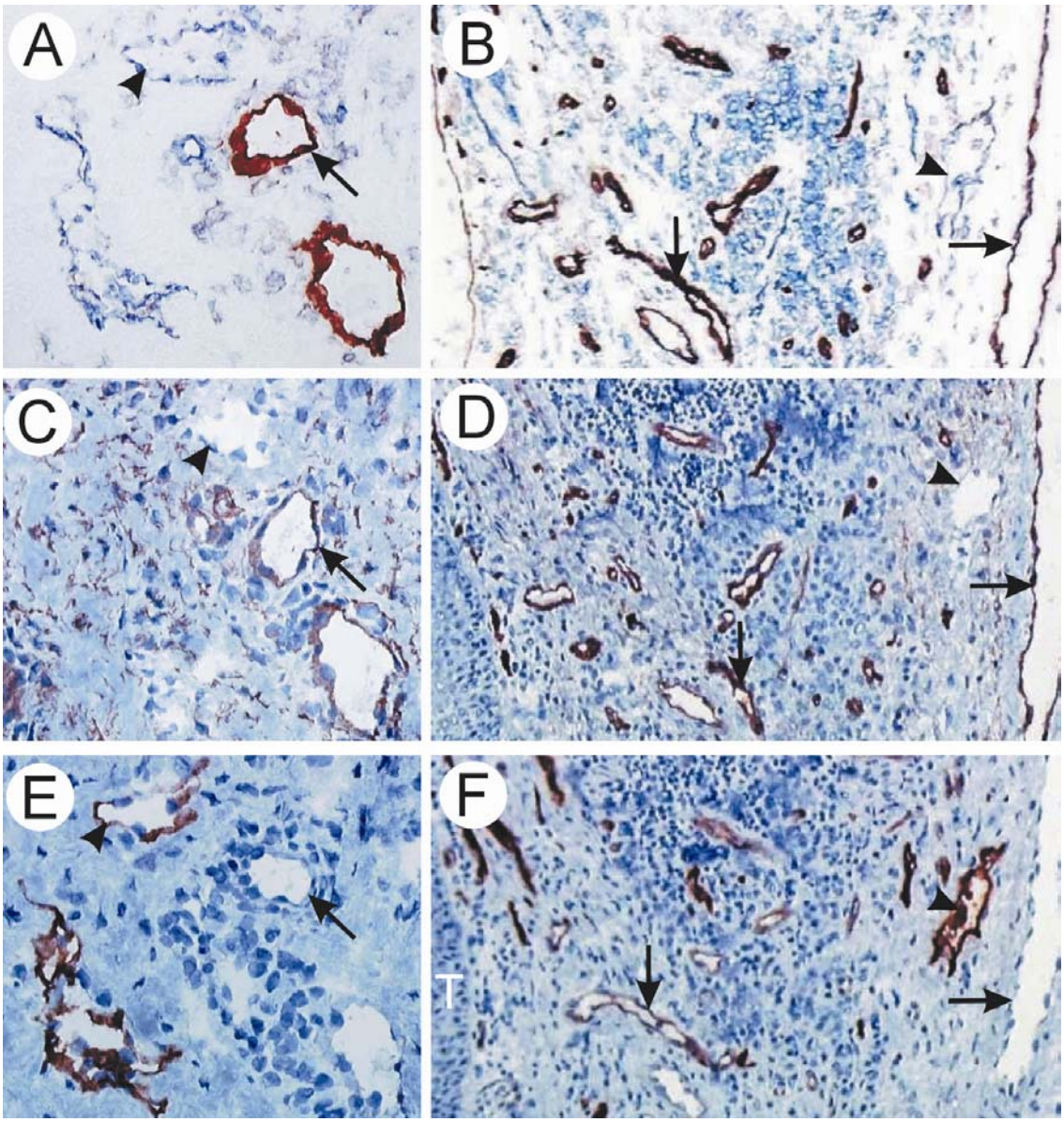
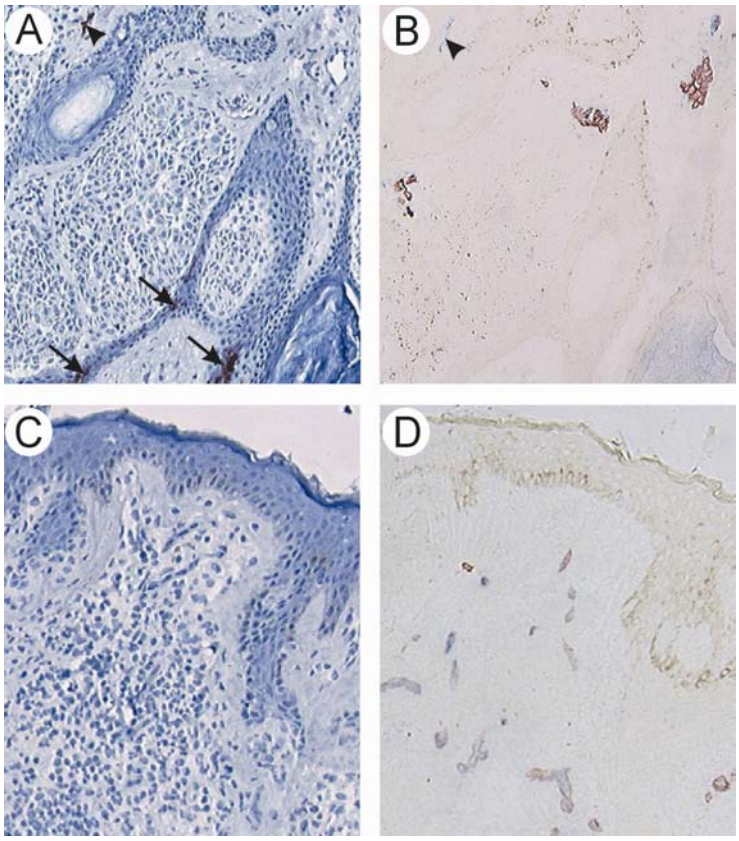
Appendix: colour figures

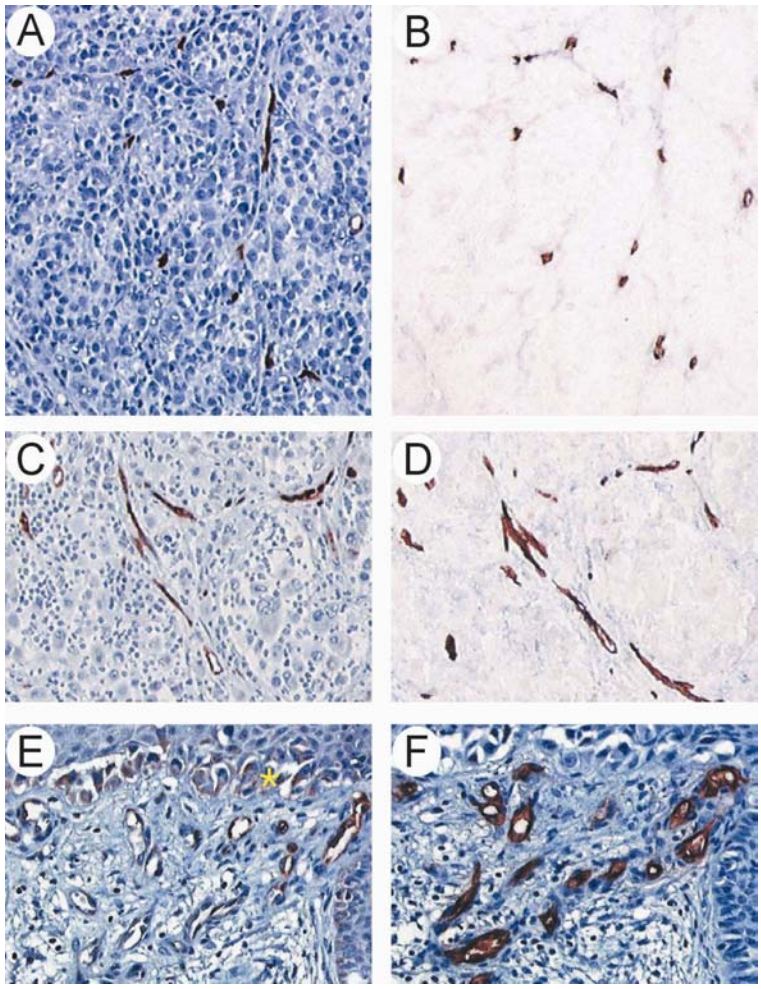


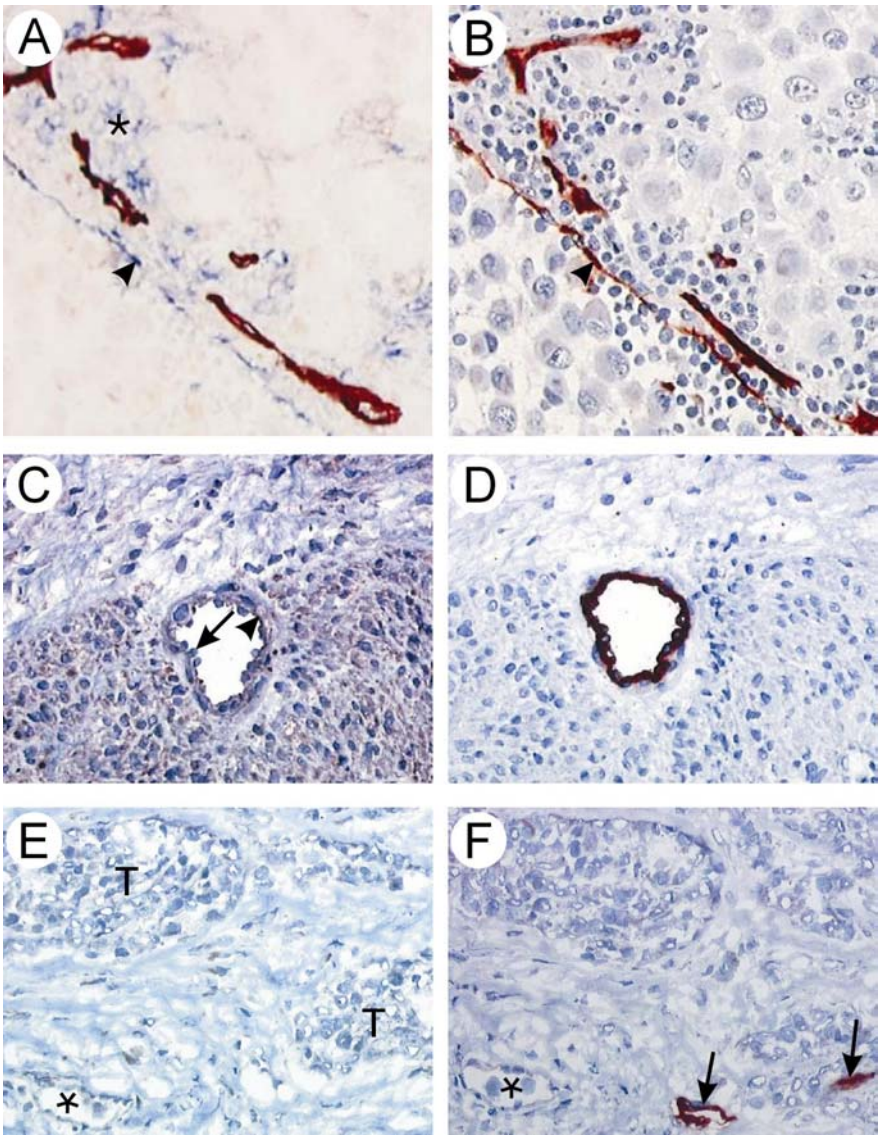
Chapter 2, figure 1 (page 28): Immunohistochemical analysis of the vasculature in serial sections of preputial skin (**a**,**c**,**e**) and of squamous cell carcinoma of the larynx (**b**,**d**,**f**) (magnification: 200x). **a**,**b**: differential staining of capillary and venous blood vessels and lymph vessels. Blood vessels (arrows) are stained red by mAb PAL-E, lymph vessels (arrowheads) and arteries blue by anti-CD31 (note that the blue blood vessel (except arteries) endothelial staining by anti-CD31 is overruled by the red staining by PAL-E [7]). **c**,**d**: adjacent sections stained with the mAb QBEnd/10 (CD34). Numerous stromal cells are CD34-positive. **e**,**f**: adjacent sections stained with the mAb 9D9 (Flt-4). No staining is observed of the blood vessel (arrow) whereas lymphatics are positive (arrowhead) in the preputial skin (**e**). Both Flt-4 positive (left arrow) near the tumor cells (T) and negative blood vessels (right arrow) at a greater distance from the tumor were observed (**f**). The arrowheads in figure **a**, **c**, **e** and **b**, **d**, **f** mark identical lymph vessels, and the arrows identical blood vessels. In **c**-**f**, the QBEnd/10 and 9D9 stainings were counterstained with Harris' haematoxylin.



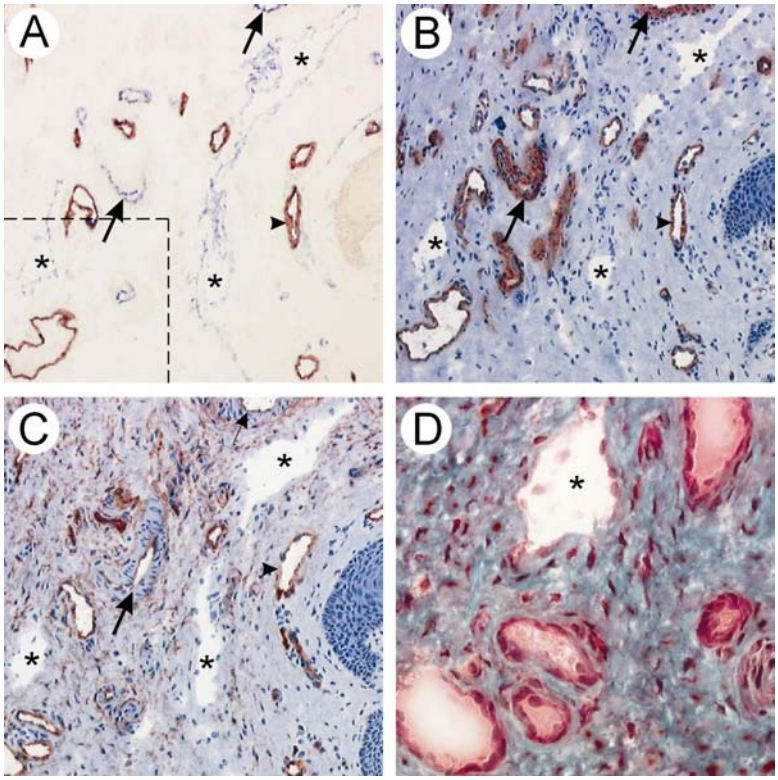
Chapter 2, figure 2 (page 30): Immunohistochemical analysis of the Flt-4 expression using the 9D9 mAb (**a**,**c**). Flt-4 expression was localized on the vasculature by comparison to a serial section stained by the PAL-E/CD31 double staining protocol (**b**,**d**) in the common acquired melanocytic nevus (**a**,**b**) and dysplastic nevus (**c**,**d**) Blood vessels are stained red by mAb PAL-E, lymph vessels and arteries blue by anti-CD31 (**b**,**d**). Flt-4 is present on lymphatic endothelium (corresponding arrowheads, a,b) whereas blood vessels are negative (**a**-**d**). Flt-4 positivity of Langerhans cells is indicated by the arrows (**a**). Sections in **a** and **c** were counterstained with Harris' haematoxylin. Magnification:100x.

Chapter 2, figure 3 (page 30): Immunohisto-chemical analysis of the Flt-4 expression using the 9D9 mAb (**a**,**c**,**e**). Flt-4 expression was localized on the vasculature by comparison to a serial section stained by the PAL-E/CD31 double staining protocol (**b**,**d**) and anti-CD34 MAb (**f**). Blood vessels are stained red by mAb PAL-E, lymph vessels and arteries blue by anti-CD31. (**b**,**d**). In primary nodular melanoma lesions (**a**,**b**) and cutaneous metastatic (**c**,**d**) melanoma lesions, Flt-4 is present on the blood vasculature. Flt-4 is expressed on blood vessels in areas of regression in a superficial spreading melanoma (horizontal growth phase) (**e**,**f**). Melanoma cells (**e**, asterisk) sometimes expressed Flt-4 as well. Sections in **a**,**c**,**e** and **f** were counterstained with Harris' haematoxylin. Magnification **a**-**b**:100x; **c**-**f**: 200x

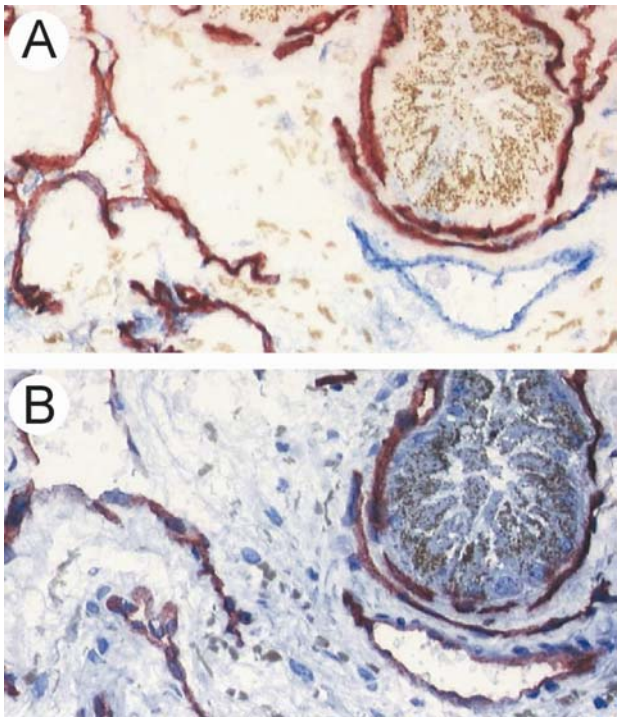




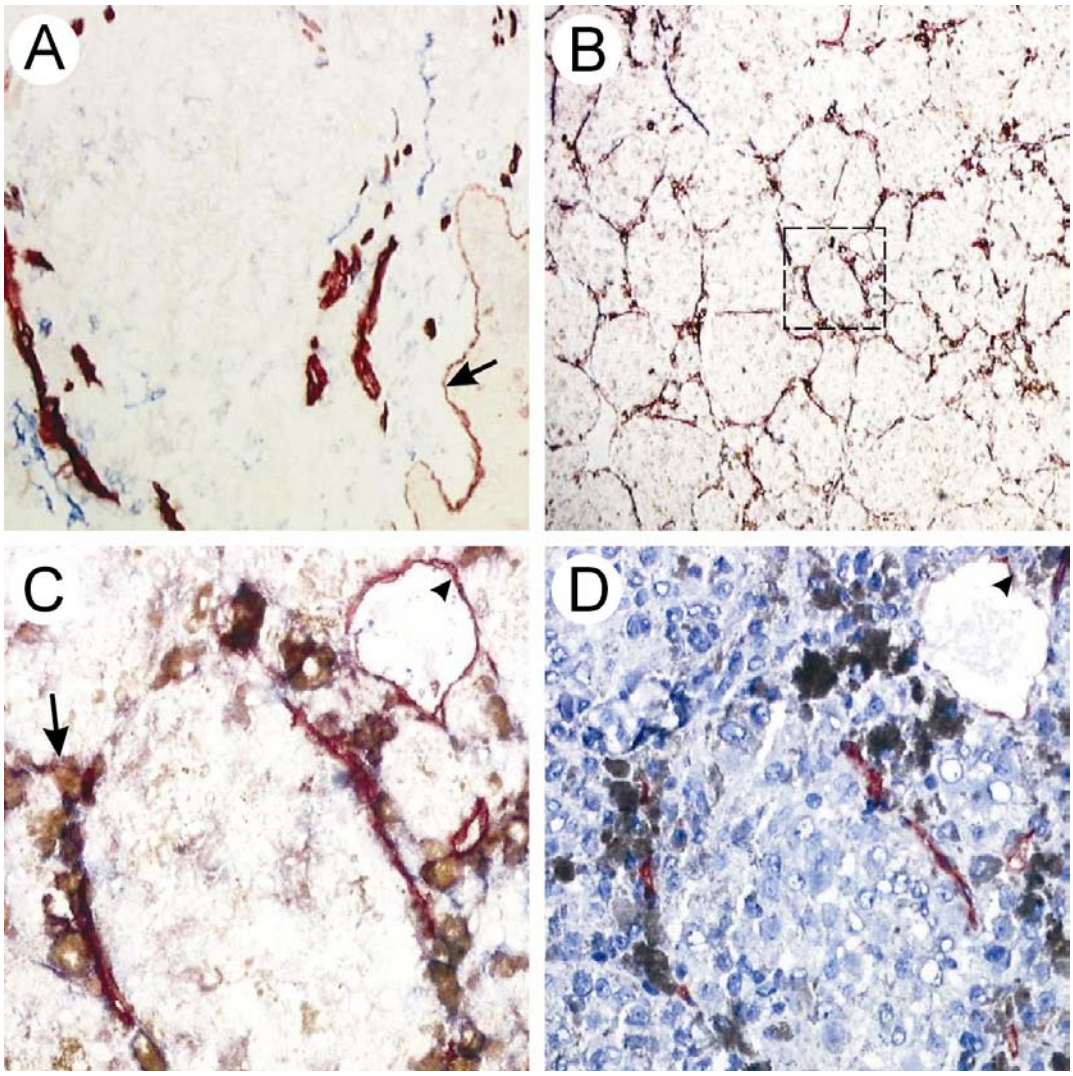
Chapter 3, figure 1 (page 42): Immunohistochemical analysis of the vasculature (**a**), Flt-4 (**b**,**d,f**) and VEGF-A (**c**,**e**) expression in a lymph node metastasis (**a**,**b**) and cutaneous metastatic lesions (**c**-**e**) of cutaneous melanoma in a human patient. Blood vessels are stained red by blood vessel marker PAL-E, lymph vessels blue by the pan-endothelial marker CD31 as previously described.[10,18] Blue blood vessel endothelial staining by the anti-CD31 mAb is overruled by the red staining by PAL-E and thereby not visible. In **a** and **b**, the lymphatic endothelial wall of the marginal sinus containing tumor cells and lymphocytes (LS, **a**) is indicated by the arrowhead. CD31-positivity was also observed occasionally on lymphocytes (*, **a**). Blood vessel in the tumor lesion were strongly positive for Flt-4 (**b**). Strong VEGF-A staining of tumor cells co-localised with VEGF-A (arrowhead, **c**) and Flt-4 (**d**) on endothelial cells. A number of cells, likely inflammatory cells, adhere to the endothelium (arrow, **c**) which are also positive for Flt-4 (**d**). In **e** and **f**, VEGF-A expressing tumor cell islets (T) are surrounded by stroma. At some distance of these tumor cells, a blood vessel (indicated by the asterisk in its lumen, **e**) is hardly positive for VEGF and Flt-4. Two lymphatic vessels are Flt-4 positive (arrows, **f**). Magnification **a**,**b**: 200x, **c**-**e**: 250x. Sections in **b**-**e** were counterstained with Harris' haematoxylin.



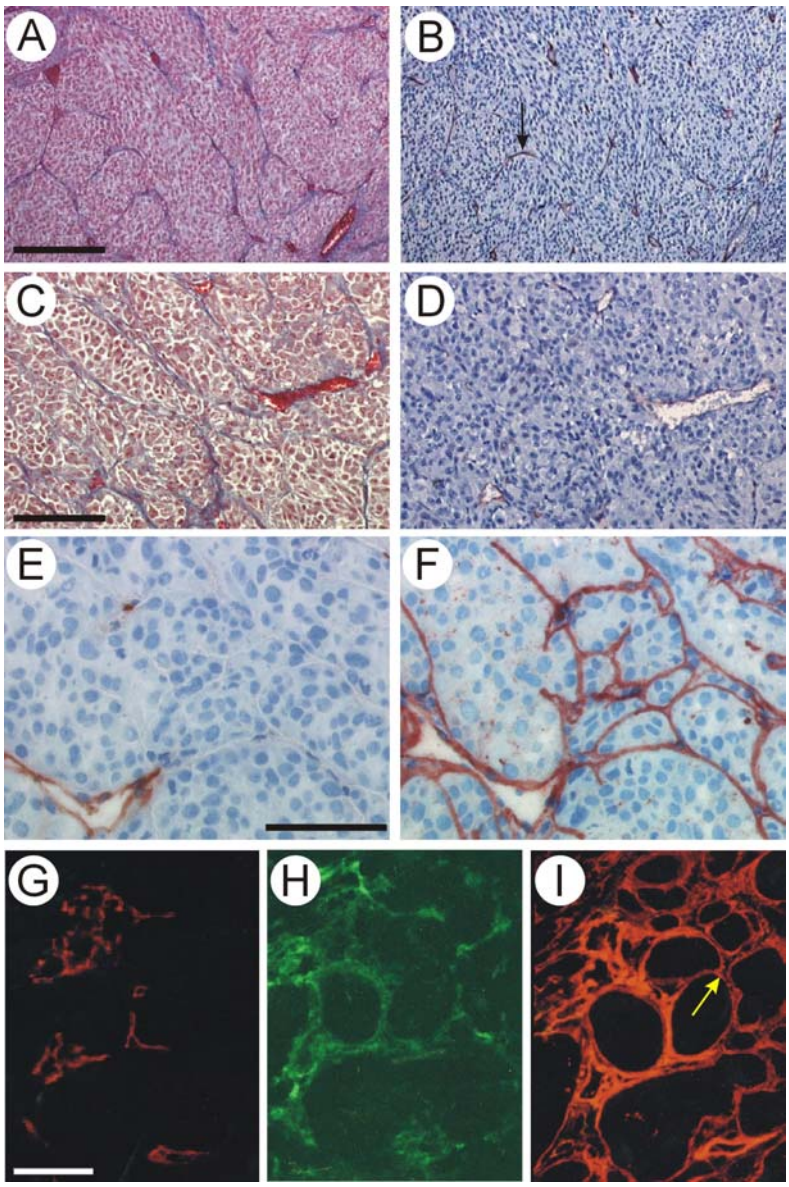
Chapter 4, figure 2 (page 54): (Immuno)histochemical analysis of the vasculature in a preputial skin section shows differential staining of blood and lymph vessels. Blood vessel capillaries and venules are stained red by the PAL-E mAb, lymph vessels and arteries blue by the anti-CD31 mAb (**a**). Corresponding lymphatics in **a**, **b** and **c** are indicated by asterisks, two arteries by arrows and a remaining vein by arrowheads. In **b**, staining by an anti-alpha-smooth muscle actin mAb demonstrated the absence of smooth muscle cells in lymphatics, whereas all blood vessels are positive. In **c**, anti-CD34 staining is selectively confined to the blood vasculature including arteries (arrows). Numerous stromal cells are CD34-positive as well. In **d**, Masson trichrome histo-chemistry of the boxed area (**a**) is depicted, showing that evident differences in vessel morphology are present (using a modified protocol, connective tissue stains green instead of blue): in the lymphatic vessel wall (asterisk) only endothelial cells are present. Stainings in **b** and **c** were counterstained with Harris' haematoxylin. Magnification **a**,**b**,**c**: 100x; d: 250x.



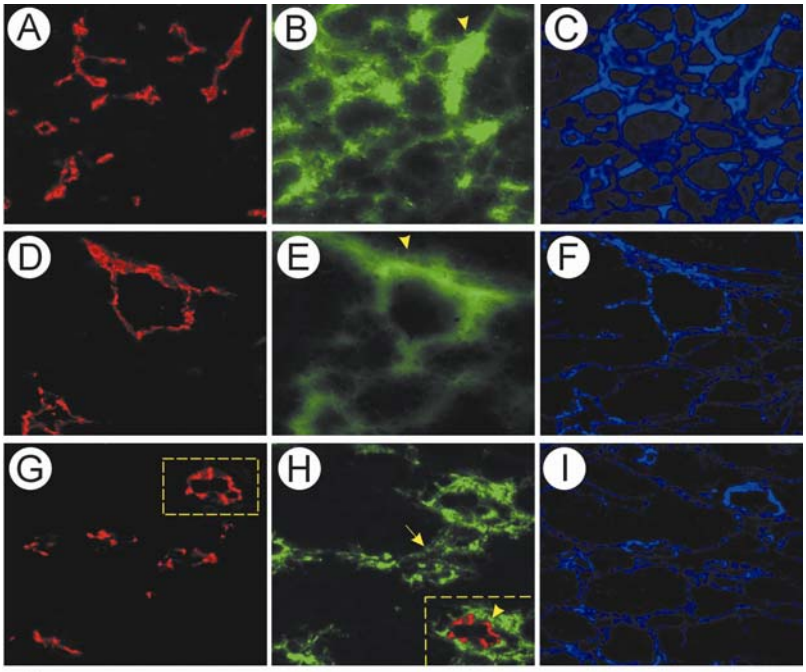
Chapter 4, figure 3 (page 54): Immunohistochemical analysis of the vasculature in serial sections of the choroidal layer of the ocular wall. **a:** differential staining of blood and lymph vessels. Blood capillaries and venules are stained red, lymph vessels and arteries blue. By comparison with the anti-CD34 staining (**b**) and on basis of previous results,[27] the blue vessel was classified as an arterial vessel. No lymphatics were present. **b:** adjacent section stained with the mAb QBEnd/10 (anti-CD34), counterstained with Harris' haematoxylin. Magnification: 200x.



Chapter 4, figure 4 (page 53): Immunohisto-chemical analysis of the vasculature in cutaneous (**a**) and uveal (**b**,**c**,**d**) melanoma. Blood capillaries and venules are stained red by mAb PAL-E, lymph vessels and arteries blue by anti-CD31 (**a**,**b**,**c**). The arrow indicates staining of the basal membrane by mAb PAL-E. No lymphatics or large arteries are present in the uveal melanoma (**b**). Details in the area marked in **b** are depicted in **c** and **d**. The vasculature in **c** is clearly stained red by mAb PAL-E. Macrophages (arrow) containing melanin are present along the vasculature. In an adjacent section (**d**), the anti-CD34 mAb produced a weaker staining signal than the PAL-E mAb (corresponding arrowheads in **c** and **d**). Magnifications: **a**: 200x, **b**: 100x and **c**,**d**: 250x. CD34 staining in **d** was counterstained with Harris' haematoxylin.



Chapter 5, figure 2 (page 67): Evaluation of extracellular matrix and blood vessels in human primary melanoma (**a**,**b**) and xenografts of cutaneous melanoma (**c**,**d**) in serial paraffin-embedded sections. Extracellular matrix is stained blue, nuclei of tumor cells red and erythrocytes intensely red by Azan histochemistry (**a**,**c**). Arcs and back-to-back loop network patterns were clearly visualized. Blood vessels were detected by CD34 (**b**) and ASD-13 (**d**) immunohistochemistry. Parts of the matrix patterns were also CD34-positive (arrow, **b**). Within the matrix patterns, no lumina were observed except where blood vessels were present. Erythrocytes were exclusively observed in association with endothelial immunostaining. In **e**-**i**, the distribution of i.v. administered tracer (FITC-BSA) in cryosections of xenografts of Mel57 melanoma cells was evaluated. In serial sections, the distribution of endothelium (**e**) and laminin (**f**) was visualized by immunostaining demonstrating a similar pattern as observed by Azan and endothelial (immuno) histochemistry in paraffin-embedded tissues (see also figure 1,3 (pages 70-71). In Mel57-xenografts, the tracer (green, **h**) is distributed in and outside the blood vasculature stained red by endothelial marker Mec 7.46 mAb (**g**), along the extracellular matrix, stained red by laminin Abs (**i**). A space between the laminin sheets surrounding coherent groups of tumor cells is indicated by the arrow. CD34, ASD-13, Mec 7.46 (**e**) and laminin (**f**) stained sections were counterstained with Harris' haematoxylin. Bar = 0.1 mm.



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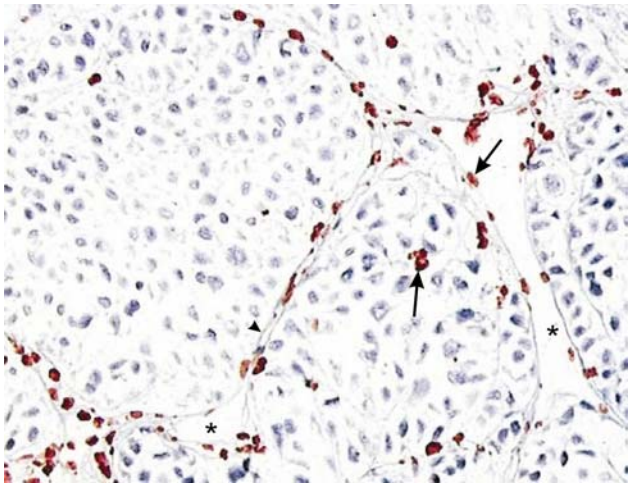
Tracer	Time after injection: 2 min	10 min	15 min	30 min	45 min	1 h	3 h	24 h
FITC-BSA	N T						N T	
FITC-I		N T	N T	N T	N T	N T	N T	N T
FITC-D20					N T	N T	N T	N T
FITC-D40							N T	N T
FITC-D70	N T							
FITC-D2.000	N T		N T		N T		N T	

Chapter 6, figure 2 (page 82): Distribution of FITC-I (**b**) and FITC-D20 at 2 min (**e**) and FITC-D2,000 at 60 min (**h**) after i.v. injection. Endothelial cells were detected by CD31 (in red: **a**,**d**,**g**) and the ECM patterns by laminin (in blue: **c**,**f**,**i**) immunofluorescence. FITC-I and FITC-D20 rapidly distributed in- and outside the vasculature along the ECM patterns. The fuzzy fluorescence pattern seen with FITC-I or FITC-D20 may indicate leakage between tumor cells outside the ECM patterns (arrowheads **b**,**e**). In **e**, tumor cell nuclei are visible (arrowhead). To show FITC-D2,000 in more detail, tracer distribution in the boxed area (**g**) containing a large vessel is depicted in the boxed area in **h** by digitally superimposing **g** on **h**. Endothelial CD31 staining in the boxed area in **e** has been superimposed on tracer staining (inset, **h**). FITC-D2,000 leaked into the perivascular space (arrowhead, **h**) whereas only minimal leakage into matrix structures occurred in the neighbourhood of major vessels (arrow, **h**). Magnification: 400x In **j**, Tracer distribution data in the course of time after i.v. injection is summarized. Dashed boxes indicate intra- or directly perivascular presence of tracer (as shown in figure 2**e**); boxes with gray to black filling indicate increasing amounts of tracer present in the ECM patterns (as shown in figure 2**d**,**e**,**g**). White boxes represent absence of tracer. NT: not tested.



Chapter 6, figure 3 (page 84): DNA in situ hybridization analysis of a melanoma xenograft lesion using total human DNA (labeled blue) and total murine DNA (labeled red) for simultaneous differential detection of human and murine cells. Murine cells were associated with the ECM patterns (arrowhead) and formed the endothelial lining of the microvasculature (asterisks). Sporadically, murine cells infiltrated inside tumor cells nests (arrow). Magnification: 400x.